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EDITED BY

SIMON FLEXNER, M.D.

PEYTON ROUS, M.D.

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No. 1. SIMON FLEXNER, J. W. JOBLING, and MAUD L. MENTEN. Experimental studies on tumor. Plates 1-28. (Issued June 30, 1910.) Price, \$1.00.

A FILTER-PASSING VIRUS OBTAINED FROM *DERMACENTOR ANDERSONI*.

By HIDEYO NOGUCHI, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, February 17, 1926.)

In the course of a bacteriological study of *Dermacentor andersoni*, the wood tick concerned with the transmission of Rocky Mountain spotted fever¹ and of tularemia,² a filter-passing virus was met with and, apparently, carried through seven generations *in vitro*, as well as through experimental animals. The history of the tick material with which these studies were made is as follows:

Source of the Virus.

The tick from which the virus was isolated was one of a lot of 51 "drag" ticks collected in Saw Tooth Canyon, Montana, in the spring of 1925 and sent me through the courtesy of Dr. R. R. Parker, of the United States Public Health Service. Four ticks of this lot, after having been kept in the refrigerator (10–12°C.) for 54 days, were placed for 48 hours at 37°C. preparatory to tests for infection with spotted fever virus, according to the procedure of Spencer and Parker.³ The tests were first made by allowing the ticks to feed for 72 hours, each on a normal guinea pig. Ticks 1, 3, and 4 became engorged with blood; Tick 2 was found dead. The guinea pig on which Tick 1 had fed developed typical spotted fever, the others remained well. The ticks were then eviscerated, the viscera were individually suspended in 4 cc. of salt solution, and 1 cc. of each suspension was injected into a normal guinea pig. The suspensions of Ticks 1 and 2 induced typical spotted fever, that of Tick 4 produced no reaction. The sus-

¹Ricketts, H. T., *J. Infect. Dis.*, 1907, iv, 141.

²Francis, E., *J. Am. Med. Assn.*, 1925, lxxxiv, 1243.

³Spencer, R. R., and Parker, R. R., *Pub. Health Rep., U. S. P. H.*, 1924, xxxix, 3027.

pension of Tick 3, which contained an unusually large number of microorganisms very similar to those found in infected ticks, induced a fever resembling spotted fever but one which conferred no immunity to the spotted fever virus. It is this infective agent existing in Tick 3 with which the present report is concerned.

Simultaneously with the injections into guinea pigs, each tick suspension was inoculated into special culture media, which are described in another paper,⁴ and duplicate series of tubes were kept at 26°C. and at 37°C. The presence of ordinary bacterial contamination was

TABLE I.

Tick No.	Result of feeding.	Result of inoculation.
1	Typical spotted fever.	Suspension (containing moderate number of organisms similar to those in suspension of Tick 3) induced typical spotted fever.
2 (found dead).	— (Perhaps never attached.)	Suspension (containing some microorganisms) induced typical spotted fever.
3	— No immunity developed.	Suspension (containing unusually large numbers of microorganisms) induced fever very similar to that of spotted fever but none of the skin lesions of the disease. Immunity test, 17 days after inoculation, negative.
4	— No immunity developed.	Suspension (in which no microorganisms could be demonstrated) produced no reaction. No immunity developed.

controlled by the use of plain broth and agar slants. The culture made with the suspension of the dead tick, Tick 2, was discarded because of mold contamination. Cultures made from the suspensions of Ticks 1, 3, and 4 were tested on guinea pigs at various intervals, beginning as early as 72 hours of incubation. The amount of the culture used for inoculation was 0.1 cc. or less and was usually suspended for convenience in 1 to 2 cc. of saline.

No fever or other reaction developed in guinea pigs which had

⁴ Noguchi, H., *J. Exp. Med.*, 1926, xliii, 515.

received the 72 hour cultures of Ticks 1 and 4. When tested with spotted fever virus 18 days after the inoculation, the animals inoculated with the Tick 4 cultures developed spotted fever, those which had received Tick 1 cultures proved immune. No real culture, however, was obtained with the suspension of Tick 1; it was completely inert when tested again 25 days later.

The cultures made with the suspension of Tick 3, however, gave rise to exactly the same type of fever in guinea pigs as had the original material. The 72 hour cultures proved active, and as time went on their activity gradually increased. As no microorganisms could be detected by dark-field examination or by any staining method, the infective agent present in the culture will be referred to here as a virus.

The virus is aerobic; it is capable of growing at 26°C. and 37°C. and has been continued in subcultures for seven generations at 37°C. It does not grow in ordinary broth or on slant agar. It produces a slight turbidity when grown in serum media containing certain carbohydrates (mixed sugars). No putrefactive odor or gas is produced. The blood and spleen,—the latter organ always much enlarged, dark red, and soft,—of guinea pigs responding to the cultures of the virus are infective, and continuous passages from animal to animal up to the 7th generation have been maintained with the material taken on the 3rd, 4th, and 5th days of fever. No microorganisms have been seen in cultures made with the blood or spleen of such animals. The virus was recovered from the blood of infected guinea pigs. It proved to be filterable through Berkefeld N filters.

Chart 1 shows the type of temperature curve observed in infection of guinea pigs with the virus under discussion. The result of an immunity test with spotted fever blood after recovery is also indicated in the chart.

EXPERIMENTAL.

Original Material.

B-C Tick 3, fed for 72 hours on guinea pig infected with spotted fever virus, Barlow-Cooper strain. 54 days later, fed on normal guinea pig 72 hours. *Result of feeding:* Animal showed no sign of infection. *Result of inoculation of suspended viscera:* Fever (Chart 1) developed 72 hours after inoculation; animal remained

febrile 7 days. Blood withdrawn at the height of fever sterile. No scrotal or ear lesions appeared, and subsequent inoculation with spotted fever virus (17 days after inoculation of tick suspension) induced the typical symptoms and lesions of the disease.

Culture Experiments.

1st generation.—A normal guinea pig received 0.1 cc. of culture (special semi-solid and slant media) kept 3 days at 26°C. after inoculation with 0.1 cc. of suspension of Tick 3. After 4 days the animal developed high temperature which continued for 4 days (105, 106, 104.5, 104.4°F.). No scrotal lesions appeared. Tested for immunity by injection of spotted fever virus (guinea pig blood) after 23 days. No protection.

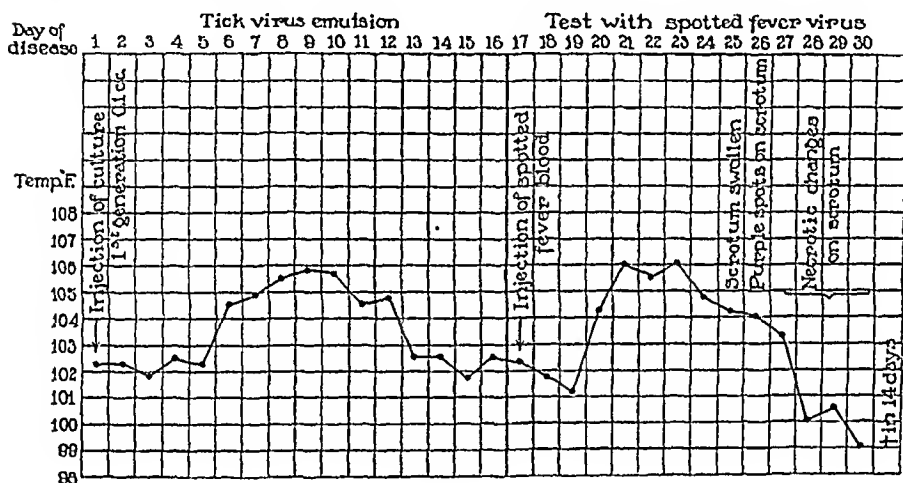


CHART 1.

Similar material, kept 3 days at 37°C., induced fever after 3 days incubation, which lasted 6 days (104.4, 105.6, 105, 103.8, 104.4, 104.2°F.). No scrotal lesions at any time. No immunity to spotted fever virus, as shown by immunity test 31 days after culture inoculation.

The same material, after 10 days at 37°C., induced a similar febrile reaction in two guinea pigs. In one, fever appeared after 4 days and lasted 6 days (104.6, 105.6, 105.6, 105.4, 105.2, 104.6°F.). No scrotal lesion developed. The animal showed no immunity against spotted fever when tested 12 days later. The second animal reacted with fever after 4 days and was killed for examination on the 5th day of fever (104.4, 105.2, 105.6, 106, 105°). The spleen was greatly enlarged and dark red, not unlike the spleen of a guinea pig infected with spotted fever; but no other lesions were found.

After 13 days at 37°C., the same material induced after 3 days incubation a

fever lasting 7 days. No scrotal lesions developed. When tested with spotted fever virus, 15 days after the inoculation of culture, this animal escaped infection. Two other guinea pigs which were inoculated with the same culture material and also reacted with fever did not prove to be immune to the spotted fever virus.

After 17 days at 37°C., the material was tested for its virulence by injecting guinea pigs with dilutions, as shown in Table II.

A second set of cultures was made with the same suspension after it had been kept 10 days at 0°C., and the culture tubes were placed at 37°C. for 8 days. A guinea pig which received 0.1 cc. of culture developed high temperature after 3 days incubation and remained continuously febrile for 8 days (104.6, 106.4, 106, 105.2, 105.6, 105, 104.8, 104.2°F.). No scrotal lesions developed at any time.

2nd generation.—Subcultures, made on the same media as the original cultures, and kept for 10 days at 37°C., were injected into two guinea pigs. High temperature developed in both animals after 6 days and was continuous in one for 6 days (104.4, 104.4, 104.6, 104.8, 104.4, 104°F.) and in the other for 5 days (105, 105.4,

TABLE II.

Amount injected.	Incubation period.	Duration of fever.	Immunity tests with spotted fever virus.
cc.	days	days	
1	3	7	No protection.
0.1	4	7	" "
0.1	3	8	Test not made.
0.01	4	7	No protection.
0.001	5	4	" "
		(105.4, 105.6, 104, 104.8°F.)	
0.0001	5	7	Test not made.
		(Chart 2.)	

105.2, 104.6, 104°). No scrotal lesions appeared. The first animal was tested for immunity to the virus 16 days after the culture inoculation. It proved immune. The second was tested with spotted fever virus after the same period. It reacted typically.

A second set of subcultures was kept 9 days at 37°C. and tested in normal guinea pigs. The animals had high temperature after 5 days which continued for 6 days (104, 104, 105.4, 105.8, 106, 104°F.). No scrotal lesions developed, and no protection against the spotted fever virus could be demonstrated. After 13 days at 37°C., the material gave rise after 3 days to high fever which lasted 7 days (104, 104, 105.5, 106.8, 107, 105.5, 104.5°), but no scrotal lesions appeared (Chart 3).

3rd generation.—Subcultures from tubes of the 2nd generation, kept 4 days at 37°C., induced in guinea pigs, after 6 days incubation, a fever lasting 5 days (104.5, 106.5, 106, 105.5, 105°F.) (Chart 4). There were no scrotal lesions. Another set of subcultures, after 8 days at 37°C., induced mild fever after 10 days incubation.

VIRUS FROM DERMACENTOR ANDERSONI

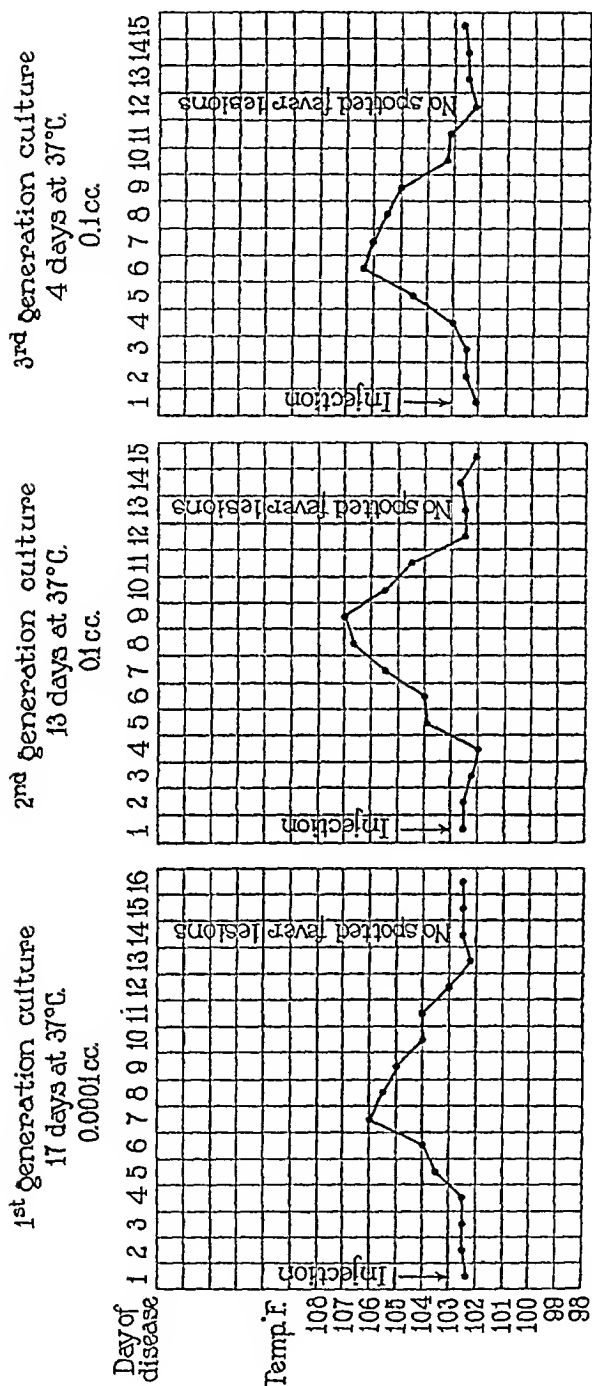


CHART 2.

CHART 3.

CHART 4.

In Charts 2, 3, and 4 are presented temperature curves of some of the guinea pigs inoculated with cultures of the 1st, 2nd, and 3rd generations.

4th generation cultures, 7 days old at 37°C., induced in one guinea pig after 10 days incubation a fever lasting 5 days, and in another fever of 3 days duration.

5th generation.—7 day old cultures, kept at 37°C., induced a febrile reaction lasting 4 days, the incubation period being 10 days.

6th generation cultures, 10 days old at 37°C., induced fever of 5 days duration after an incubation period of 7 days.

7th generation.—Subcultures from the 6th generation, after 4 days at 37°C., induced fever of 5 days duration following an incubation period of 10 days.

The number of positive reactions became less constant with further subcultures, the virus becoming gradually attenuated.

Macacus rhesus 1.—The monkey received an intraperitoneal injection of 1 cc. of mixed cultures of the virus, including one of the 1st generation (28 days old) and one of the 4th (8 days old). A fever fluctuating between 104°F. and 104.8° developed after 3 days and lasted 6 days. No spotted fever lesions appeared in the animal.

Guinea Pig Passages.

1st passage.—Guinea Pig XVIII-84B developed fever (104–105.5°F.) 5 days after receiving a 1st generation culture (13 days old at 37°C.), the temperatures recorded on successive days being 105.2, 105.4, 106, 105.5°F. The animal was killed on the 5th day of fever. The spleen was enlarged, and its appearance suggested that of the spleen in spotted fever infection. The blood, which was free from contaminating microorganisms, as tested by cultures, was injected intraperitoneally into two normal guinea pigs.

2nd passage.—Guinea Pig XVIII-121A⁵ developed fever after 4 days which continued for 7 days (106.2, 105.4, 104.6, 104°F.). Guinea Pig XVIII-121B also showed high fever after 4 days (105.6, 106.4, 105.4, 105.4°F.). It was killed on the 4th day of fever for passage, cultural experiments, and virulence tests on monkeys, rabbits, and guinea pigs.

3rd passage.—Citrate blood from the animal just mentioned was injected into four guinea pigs, the first receiving 1 cc., the second 0.1 cc., the third 0.01 cc., and the fourth 1 cc. The fourth guinea pig received in addition 2 cc. of serum from rabbits immunized to spotted fever, of a titre such that 0.1 cc. neutralized 100 M.L.D. of spotted fever virus (guinea pig blood). The incubation period in each instance was 7 days, and the duration of fever was 2 to 3 days in the first three animals and 6 days in the fourth. There was no evidence of any neutralizing effect on the virus by spotted fever antiserum.

⁵ Twenty-two ticks were placed on this animal on the 3rd day of fever and allowed to feed 5 days; nineteen became engorged, three were found dead.

The same blood was injected into *Macacus rhesus* 2 and a rabbit intraperitoneally, each receiving 2 cc., but no reaction followed in either instance.

After the 3rd passage, successive transfers were made as routine, two guinea pigs being used in each passage, with two sets of cultures set up for each, until the 8th passage, 3 months after the 1st, when the work was discontinued. No increase in virulence was noted, but on the contrary the duration of fever tended to become shorter and the incubation period longer. At each passage the blood was tested for ordinary bacterial invasion, as for example by bacilli of the paratyphoid group; no such invasion was discovered.

Filtration Experiments.

Cultures of the 1st, 2nd, and 3rd generations, grown on the special semisolid and slant media, were pooled and diluted with an equal volume of 0.9 per cent salt solution. The suspension, which contained no demonstrable microorganisms, was thoroughly shaken and passed through a Berkefeld filter N. The clear, light brownish filtrate was injected into four guinea pigs in amounts of 4, 3, 2, and 1 cc., respectively. The first three animals developed, after 4 days incubation, typical fever (104.5–105.5°F.) which was continuous for 7 days; the guinea pig which was inoculated with 1 cc. showed slight fever for 2 days. These animals were tested for immunity by injections of virulent spotted fever blood, but all had typical severe infections.

A guinea pig which had been injected as control with 1 cc. of the unfiltered suspension became ill in 4 days and passed through a course of fever lasting 5 days (106.2, 105.6, 106, 104.6, 104.6°F.).

Subcultures made with the filtrate yielded an active culture, which was subsequently tested for filterability, with positive results. Neither filtrate nor cultures contained any demonstrable microorganisms.

The question arose whether the infective agent revealed by the inoculation of guinea pigs had not merely survived in the culture tubes at 37°C. The original suspension, preserved at 3°C., was tested in guinea pigs in various dilutions on the 7th and 17th days after its preparation. The results, which are recorded in Table III, show that 1 cc. of the original material induced infection, but not 0.1 cc. or less. The culture of the 1st generation, on the other hand, when 17 days old was effective in a dose of 0.0001 cc.

As has already been mentioned, the original suspension contained an enormous number of minute, non-motile, Gram-negative microorganisms resembling spotted fever organisms. These did not grow on the media employed for cultivating the filterable virus under discussion.

Transmission of the Virus from Infected Guinea Pigs to Ticks and Vice Versa.

Experiment 1.—Seven ticks which had been fed for 5 days on a guinea pig in the febrile stage of the virus infection were placed separately on seven normal guinea pigs and allowed to feed for 5 days. In one of the animals fever developed after 8 days (104–105°F.) and lasted 4 days. The others escaped infection.

The tick which had induced infection was killed 119 days later and a portion of the emulsified viscera inoculated into a guinea pig. Fever (104.5–105.5°F.) developed after 48 hours and lasted 8 days. Death occurred on the 11th day. There were no lesions of spotted fever. The spleen was soft and considerably enlarged. No bacteria could be demonstrated in cultures made from the blood and spleen. Inoculation of blood withdrawn during the febrile stage induced the usual prolonged febrile, non-fatal reaction in guinea pigs; no spotted fever lesions developed.

TABLE III.

Amount of tick suspension.	Kept 7 days at 3°C.	Kept 17 days at 3°C.
cc.		
1	Usual fever.	Usual fever.
0.1	No reaction.	No reaction.
0.01	" "	" "
0.001	" "	" "

Smears of the tick revealed only a few microorganisms, but sections showed a large number of minute Gram-negative elements in the lumen of the intestine as well as in the viscera. Cultures made with the suspension yielded no growth.

Experiment 2.—A female tick, which had failed to induce infection by biting, survived 110 days and laid several lots of eggs. The tick and 40 of the eggs were separately suspended in saline, and a portion of each was injected into guinea pigs. No fever developed. In the smears and sections of the tick and eggs were found a number of Gram-negative microorganisms, and cultures yielded a growth of *B. rickettsiformis*.⁴ The finding was of interest in showing hereditary transmission of this non-pathogenic microorganism, which had already been found in the ovaries and egg cells of ticks.

Experiment 3.—Four ticks of a lot of eleven which had been fed for 5 days on a guinea pig infected experimentally were still living after 124 days and were separately fed on guinea pigs, but no fever was induced. The remaining seven ticks were dead but only partly dried, and a single suspension in salt solution was made of their viscera. The guinea pig injected with a portion of this suspension developed after 8 days incubation a fever of 5 days duration. The blood withdrawn on the 4th day of fever contained no contaminating bacteria. The filtrate of the emulsion failed to induce infection.

This survival of the virus in ticks for several months at refrigerator temperature is not surprising, since it occurred also in culture. A guinea pig receiving 0.2 cc. of 1st generation culture of the virus, preserved at 2°C. for 5 months, developed the typical febrile reaction after 4 days and remained febrile for 6 days, when it was killed for transfer and culture. There was no bacterial invasion.

The foregoing experiments show that the virus is taken up by ticks from the blood of the infected guinea pig, but that transmission of the infective agent to other guinea pigs by the bite of infected ticks seldom occurs under the conditions described. The presence of the virus in ticks can be demonstrated more definitely by inoculation of guinea pigs with tick emulsions. That all ticks which are fed on infected guinea pigs do not take up the virus, however, is shown by the negative results in some instances.

Cross-Neutralization Experiments.

It has been repeatedly noted that guinea pigs which have responded to the virus under discussion do not acquire immunity against the spotted fever virus. Conversely, guinea pigs which have passed through the spotted fever infection were found to be quite as susceptible to this virus as normal animals. A potent spotted fever immune serum failed to neutralize the cultured virus.

SUMMARY.

An invisible, filter-passing virus, pathogenic for the guinea pig, and capable of cultivation on special media for at least seven generations, has been isolated from a tick of the species *Dermacentor andersoni*. One of two monkeys (*Macacus rhesus*) inoculated became infected, and in one rabbit the result was negative.

The virus has been transmitted from infected guinea pigs to ticks as also in one instance by tick feeding from an infected tick to a guinea pig. The presence of the virus in the tick is more easily demonstrated by the inoculation of guinea pigs with a suspension of the tick viscera. Continuous high fever (104.5–106.5°F.) and enlargement of the spleen are the chief symptoms of the infection in guinea pigs. After the febrile attack the guinea pigs are not susceptible to reinoculation with the cultured virus but are subject to infection with the virus of spotted fever, and *vice versa*.

THE ORGANISM ASSOCIATED WITH SPECIFIC INFECTIOUS CYSTITIS AND PYELONEPHRITIS OF COWS.

By F. S. JONES, V.M.D., AND RALPH B. LITTLE, V.M.D.

(From the Department of Animal Pathology of The Rockefeller Institute for Medical Research, Princeton, N. J.)

PLATE 1.

(Received for publication, March 31, 1926.)

In a previous communication¹ we described a specific infectious cystitis and pyelonephritis of cows apparently not observed previously in this country. Owing to the inclusion of cases of other types of infections of the kidneys in the reports of certain writers and the slight comment on the bladder lesions, considerable confusion seems to have arisen both in regard to the organism and its method of reaching the kidney. The organism most frequently obtained in Europe thus far has failed in the hands of several observers to reproduce the disease.

Höfllich² and Enderlen³ cultivated from affected kidneys a Gram-positive diphtheroid. Enderlen named it *B. pyelonephritis boum*. Ernst,⁴ who obtained pathologic kidneys from 24 cows slaughtered at the abattoir, found diphtheroids similar to those cultivated by Höfllich and Enderlen in 11 instances. The others were associated with *B. pyogenes*, *B. coli*, and the tubercle bacillus. These workers agree that the diphtheroid is not pathogenic for the smaller animals. They obtained only negative results by intravenous injection of cultures into cows. Ernst first injected sterile sand into the bladder of a cow and on the following day 100 cc. of broth culture. Violent inflammation ensued but subsided after 9 days. This experiment, with the negative experiments reported by himself and others, led him to question the relationship of the diphtheroid to pyelonephritis.

In our first communication little was reported concerning the bacteriological findings. In all our cases we were able to readily cul-

¹ Jones, F. S., and Little, R. B., *J. Exp. Med.*, 1925, xlii, 593.

² Höfllich, D., *Monatsch. prakt. Tierheilk.*, 1891, ii, 337.

³ Enderlen, D., *Z. Tiermed.*, 1890-91, xvii, 325.

⁴ Ernst, W., *Centr. Bakt., 1. Abt., Orig.*, 1905, xxxix, 549, 660.

tivate a diphtheroid from the urinary sediment and from the pelvis of affected kidneys. As a rule the cortex was not invaded by the bacillus although in a few instances it was obtained from cortical lesions of advanced cases. The bacilli were usually found in practically pure culture, but at times cocci and streptococci were encountered in small numbers. From the morphology and lack of pathogenicity for small animals as noted by Enderlen, Höflich, and Ernst, it is evident that our cultures resemble those obtained by these workers. From 6 cases we cultivated the organism from the kidney. From the urine of 20 definitely affected cases the same organism was obtained. In the main it is true that the bacilli usually occur in the pelvis of the kidney in pure culture. They always predominate in the urine and frequently occur in pure culture, although at times cocci of various sorts are found. These bacteriological findings associated with lesions of uniform character led us to conclude that we were dealing with a specific infectious disease.

The Morphological and Cultural Characters of the Organism.

The bacillus is non-motile and in the urinary sediment is found in dense masses. The rods are slender and vary from 2 to 3μ in length. They stain well with the ordinary stains and are Gram-positive. Those from the tissues are not as pleomorphic as those from the earlier transfer cultures, although many may show polar granules or swollen ends. The morphology of cultures grown in broth is more variable; short coccoid forms, beaded rods with swollen ends, as well as the usual slender rods are abundant (Fig. 1). Occasionally a stalk with rudimentary branches may be observed.

The bacillus is readily grown on ordinary media. In liquids, such as broth or urine, aggregates of considerable size are found at the bottom of the tube. On the surface of agar or serum the growth is raised, grayish white, and dry. On potato the growth at first is usually grayish white, but later it becomes a dingy yellow and the potato turns brown. Blood serum and gelatin are not liquefied and hemolysin is not produced. In litmus milk the growth reaction is characteristic. The litmus at the bottom of the tube is reduced, here the casein coagulates, but the upper stratum is deep blue and coagulates slowly. The casein is digested slowly. Of the carbohydrates tested only dextrose has been attacked. No gas is produced, but a final hydrogen ion concentration of pH 4.9 to 5.0 is a regular finding. Lactose, saccharose, maltose, and mannitol are not attacked, since only alkali is produced in tubes containing them. These findings were constant for the 26 strains studied.

TABLE I.
The Agglutination Affinities of Various Strains of the Diphttheroid Organism.

Case No. and source of the strain.	Antiserum Strain 1132 (kidney), Herd A.						Antiserum Strain 1143 (kidney), Herd B.						Antiserum Strain 1151 (urine), Herd B.					
	Dilutions of serum.						Dilutions of serum.						Dilutions of serum.					
	1:20	1:50	1:100	1:200	1:500	1:1,000	1:20	1:50	1:100	1:200	1:500	1:1,000	1:20	1:50	1:100	1:200	1:500	1:1,000
1132. Kidney. Herd A.	C.*	C.	+++	+++	+++	+	C.	C.	C.	C.	+++	+	C.	C.	+++	++	+	+
273. Urine. Herd A.	C.	C.	C.	+++	+	+	C.	C.	C.	C.	++	+	C.	C.	C.	++	+	+
139B. Urine. Herd A.	C.	C.	C.	+++	+	+	C.	C.	C.	C.	++	+	C.	C.	C.	+++	+	+
1143. Kidney. Herd B.	C.	C.	C.	+++	+	+	C.	C.	C.	C.	+++	+	C.	++	+	+	+	+
1151. Urine. Herd B.	C.	C.	C.	C.	+	+	C.	C.	C.	C.	+	+	C.	C.	C.	++	+	+
1154. Urine. Herd B.	C.	C.	+++	+++	+	+	C.	C.	+++	+++	++	+	C.	C.	C.	++	+	+
1243. Urine. Herd B.	C.	C.	C.	+++	+	+	C.	C.	C.	C.	+	+	C.	C.	C.	++	+	+
1223. Urine and kidney. Herd C.	C.	C.	+++	+	+	+	C.	C.	C.	++	+	+	C.	C.	C.	+	+	+
1225. Urine. Herd C.	++++	++++	++	+	+	+	++++	++	++	+	+	+	++++	++	++	++	+	+
K.M. Urine. Herd D.	C.	C.	C.	+++	+	+	C.	C.	C.	++	+	+	C.	C.	C.	+++	+	+
K. Urine. Herd E.	++++	++	++	+	+	+	++++	++	++	++	++	+	++	++	++	+	+	+

* The degree of agglutination has been recorded as follows: C., complete clearing of the fluid as result of agglutination; ++++, a heavy clumping without complete clearing; ++, well defined clumping; +, less well defined clumping; ±, a definite deposit on the bottom of the tube; -, a slight deposit on the bottom of the tube.

Since it was possible to show that we were dealing with a group of organisms whose morphological and cultural characters were identical, it was desirable to know whether strains from different herds were immunologically related.

Our material was drawn from five herds in this vicinity and, since there was no exchange of cows between the herds, we were presumably dealing with fairly independent strains. To establish their immunological relationship, rabbits were immunized with cultures of the various strains. After the serum had reached a maximum agglutinin titer the animals were bled. The serum was tested against salt solution suspensions of all the cultures. The tendency of certain strains to clump spontaneously was largely overcome by rapid transfer on artificial media and mechanical shaking of the suspension with glass beads. Inasmuch as the 26 strains reacted to about the same degree, the details concerning only a few from each herd are given in Table I.

In the main it can be said that all cultures reacted to a greater or less degree to three agglutinins prepared by immunizing rabbits with single strains. It is of further interest to note that those isolated from the kidneys at autopsy or from the urine of cows in Herds A, B, C, and D, behaved in an almost identical manner to all agglutinins. The strain from Herd E also agglutinates with the specific sera but to a less degree.

Pathogenicity for Animals.

Our cultures, like those described by the European workers, are not pathogenic for rabbits or guinea pigs. Rabbits easily withstand an intraperitoneal inoculation with 10 cc. of a 48 hour bouillon culture. Guinea pigs and white mice are equally resistant. We were unable to show that the organism produces a soluble toxin. Young guinea pigs injected intraperitoneally with filtrates of unheated 48 hour and 5 day broth cultures fail to react. In our first communication mention was made of experiments in which broth cultures filtered through Berkefeld candles were injected into and beneath the skin and instilled on the conjunctiva of infected cows. In no instance were allergic reactions obtained.

A number of experiments were made with cows. In two instances cows were injected intravenously with 5 cc. and 10 cc. of freshly isolated broth cultures. Slight temperature reactions occurred shortly

after the injections, but the organism never appeared in the urine. At autopsy the urinary system failed to show abnormalities. On two occasions sterile swabs moistened with cultures were introduced into the vagina. The organism failed to develop in the vagina, and as far as we could determine the bladder never became invaded. The findings at autopsy were negative. In two instances cows were fed large quantities of freshly isolated broth cultures. One developed typical cystitis associated with the diphtheroid. The other remained normal. The experiment was repeated with entirely negative results. The feces were examined a number of times after the cows had been fed the cultures but the organism could not be found.

Instillation of small quantities of culture into the bladder led to definite infection. Since this is the first recorded experiment in which infection with pure culture of the diphtheroid has been established the details are given.

Cow 1100.—A Holstein that had been under observation at the Institute for over 10 months. Samples of urine were obtained for examination on several occasions before the injection. The urine was normal and contained only a few cocci. On Jan. 24, 1925, the bladder was emptied by means of a catheter and 3 cc. of a 24 hour broth culture in the 3rd generation was introduced. On Jan. 26, the urine contained a little albumen; the sediment contained a few red blood cells, leucocytes, cocci, and diphtheroids. The next day the bacilli were much more numerous, but the urine presented no gross abnormalities. On Jan. 28, the sample was slightly turbid. It reacted to HNO_3 . The sediment contained epithelial cells, leucocytes, and bacilli in enormous numbers. Plate cultures contained the organism in practically pure culture. On this day the cow strained considerably at the close of urination. Daily examinations were made until Feb. 4. On this day the urine was dark brown in color. It reacted markedly with HNO_3 and gave an Esbach reading of $\frac{1}{4}$ per cent albumen. There was considerable sediment composed of red cells, leucocytes, and large numbers of bacilli. For the next 3 days red cells were always observed in the sediment. From this time until Mar. 10 the urine was examined daily. It always reacted to tests for albumen. The amount and character of the sediment varied. At times red cells made up the bulk of it, at others leucocytes predominated. The bacilli were always present in large numbers (Fig. 2). On Mar. 11, 1925, the cow was slaughtered at an abattoir. The kidneys and ureters presented no gross abnormalities. The bladder, however, showed characteristic lesions (Fig. 3). The mucosa of the ventral half was swollen. It was thrown up into broad raised folds. The color of the involved portion was yellowish pink and mottled with scattered, well defined, irregular, deeply reddened areas. Under slight mag-

nification the reddened areas were composed of points, lines, and patches of deep red extravasations located within the mucous membrane. The bacilli were readily cultivated from the mucosa overlying the congested areas. Microscopic examination of fixed and stained sections from the kidney and ureter failed to show abnormalities. Those from the bladder revealed definite lesions. The mucosa was degenerated. The nuclei were swollen and stained faintly; many were observed to lie within a clear vacuole. In certain instances the nuclei were shrunken, granular, and stained deeply. Beneath the mucosa there was a band of fibrinous exudate containing many round cells and a few leucocytes. The submucous connective tissue was edematous. Aggregates of round cells were scattered throughout the submucosa; they were particularly numerous just beneath the mucosa. The edema extended into the muscular coat.

Another cow was inoculated into the urethra with 5 cc. of broth culture. Albumen appeared in the urine 3 days after injection and the bacilli were present in the sediment in practically pure culture. The cow was under observation for over 3 months. The urine was examined frequently. The bacilli were always present. Albumen could usually be detected; leucocytes and red cells were usually present in the sediment and at times in considerable quantities. The cow was slaughtered 100 days after the inoculation. The kidneys and ureters failed to show gross changes. About five-eighths of the mucosa of the bladder was swollen. It was thrown up into broad folds and rounded hummocks. The involved portions varied from yellow to orange in color and were sprinkled with red areas varying from points barely visible to hemorrhages 1.5 to 2 cm. in diameter. Microscopic examination of fixed and stained sections (Fig. 4) revealed degeneration of the mucosa, hemorrhage into the mucous layer, accumulations of round cells and leucocytes just beneath the mucosa, and numerous accumulations of round cells scattered throughout the edematous submucosa.

From our experiments it becomes clear that the bacillus is not pathogenic when introduced into the blood stream. In our first communication we placed particular emphasis on the bladder lesions, since cystitis was observed in all "spontaneous" cases of the disease. Instillation of pure cultures of the bacilli obtained from the urine or kidney into the bladder of normal animals results in a well defined cystitis. It is true that the cystitis encountered under natural conditions is more severe, but the character of the urine and the gross and microscopic pathology are the same.

Ritzenthaler⁵ called attention to the fact that the means by which cows become infected is unknown. We attempted to throw some light on this phase of the problem. Since we recognized that the

⁵ Ritzenthaler, M., *J. Comp. Path. and Therap.*, 1910, xxiii, 33.

process is an ascending one, it seemed possible that the organism might inhabit the vagina of the normal cow or that it might be associated with metritis. The vaginal secretions of a considerable number of normal cows were examined bacteriologically. Diphtheroids were found, but in no instance could it be said that an organism identical with the pyelonephritis and cystitis bacillus was cultivated. The experimental inoculations of culture into the vagina confirmed these observations, for it was shown that the organism soon died when implanted on the vaginal mucosa.

It is conceivable that the bull during copulation might readily transmit the organism. The urine from 11 bulls was collected in rubber bags and examined. Diphtheroids were encountered in several samples but all differed culturally and immunologically from the specific bacillus. It seemed possible that the penis of the bull might become contaminated with the bacillus and thus the organisms gain access to the vagina of other cows. From the herd records we were able to obtain the dates on which known cases of the disease were bred, together with the record of the males' subsequent breedings. In this way we found three bulls that had bred 26 cows shortly after copulation with an infected cow. Urine examinations of these cows showed that one was infected. This cow occupied for several months an adjoining stall to a severe clinical case of the disease. In an isolated observation one bull served a cow suffering from a well marked cystitis. Within the next half hour the bull served three other cows. One of the cows was disposed of shortly after the breeding, but the other two remained uninfected. It is clear then that the bull is not a ready agent of transmission.

It is customary in the herd where most of our observations have been made to curry and brush the cows. Vigorous currying and brushing of the regions about the anus and vulva are done twice daily. This suggested itself as a means of transmission, especially since the bacilli might be implanted in tiny wounds or in exuded urine adhering to the vulva. An opportunity was afforded for observations on this point. Well defined cases of the disease were placed among four uninfected cows in such positions that the infected cows were brushed first. Two cows were disposed of within 1 month of exposure. From the urine of the third the bacilli were

isolated. This cow recovered. The fourth cow was found infected 5 months after the exposure was started.

DISCUSSION.

We concur with the opinion expressed by Joest⁶ that pyelonephritis of cows is a specific infectious disease. The organisms that we have encountered in cases occurring on five different farms have been identical in morphology and cultural characters. This organism when introduced in small quantities into the bladders of normal cows promptly sets up cystitis which becomes chronic. The experimental disease is on the whole milder than that usually encountered in the herd, but the bacilli will persist in the urine for a period of months. In the case of the cows of our experiments it must be borne in mind that the animals were not pregnant and were fed only a moderate grain ration. Many have noted that certain forms of nephritis in cows may occur shortly after parturition. We have previously pointed out that statistics in the matter are liable to considerable error because of the possibility that there may have existed a long standing but unnoticed infection. However, we are inclined to believe that pregnancy may play a considerable part in increasing the severity of the disease. During the dry period the concentrates in the ration are diminished and a considerable bulk of dry matter is substituted. The amount of urine excreted is considerably less. The organisms will grow readily in normal cow's urine and abundantly in urine containing a little blood serum. Under the conditions of pregnancy, then, there is an ample opportunity for the bacilli to multiply and exert a relatively prolonged action on the bladder mucosa. Late in pregnancy the pressure on the bladder, and perhaps injury to the bladder during parturition, may serve to influence the disease unfavorably.

In certain respects the causative organism is unique. It possesses no pathogenicity for the smaller animals. As far as we can learn it inhabits only the urinary tract of cows, unlike other organisms associated with renal infection, such as *Bacillus coli* and *Bacillus pyogenes*. The organism cannot be said to be an inhabitant of the

⁶ Joest, E., *Spezielle pathologische Anatomie der Haustiere*, Berlin, 1924, iii.

normal vagina or uterus and, as far as can be learned, plays no part in metritis. In our experiments in which large quantities of broth cultures were fed to cows, the bacilli never appeared in the feces. The lymph glands of the head and neck of a cow fed the organism were cultured with negative results. On the whole it can be said that it possesses but little pathogenicity for cows unless it finds its way into the urine where it readily grows and attacks the structures lining the bladder. The large numbers of bacilli in the urine in even mild cases strengthen the view that this is the method whereby the injury comes about.

Our experiments have thrown little light on the mode of dissemination of the organism. However, we feel that certain portions of the work are suggestive. In the first place, the bacilli attack only the mucosa of the urinary tract. They are not concerned in metritis. The disease is apparently independent of genital conditions. Once the bacilli gain access to the bladder they multiply and produce the characteristic cystitis. We feel certain that they do not reach the bladder by way of the blood stream, since cows injected intravenously with culture failed to develop cystitis. The experiments in which small amounts of culture were introduced into the vagina were negative. None of the cows became infected. Nevertheless, under most favorable conditions, organisms deposited in the vagina in the region of the urethra might very well find a favorable medium for growth and thence reach the bladder. When intimate contact occurs over long periods it has been possible to observe transient and prolonged infection, as in the experiment in which cows were placed in stalls adjoining clinical cases of the disease. Under these conditions the cows were curried and brushed vigorously twice a day and doubtless as a result the bacilli were mechanically deposited in the vagina at frequent intervals. From our observations we feel that the bull does not often act to disseminate the disease during copulation. Males are relatively insusceptible to the disease.

SUMMARY.

A Gram-positive diphtheroid isolated from spontaneous cases of cystitis and pyelonephritis of cows on five farms has been studied.

All the strains obtained were of the same general morphology. They possess similar cultural characteristics and to a great degree similar agglutination affinities. The organism is not pathogenic for the ordinary laboratory animals. It produces no toxin. Cows injected intravenously with broth cultures failed to develop the disease. On the other hand, small quantities of cultures introduced into the bladder by way of the urethra gave rise to a typical persistent cystitis.

There is evidence for a transmission of the disease by contact infection, as when the same individual carries both normal and infected cows.

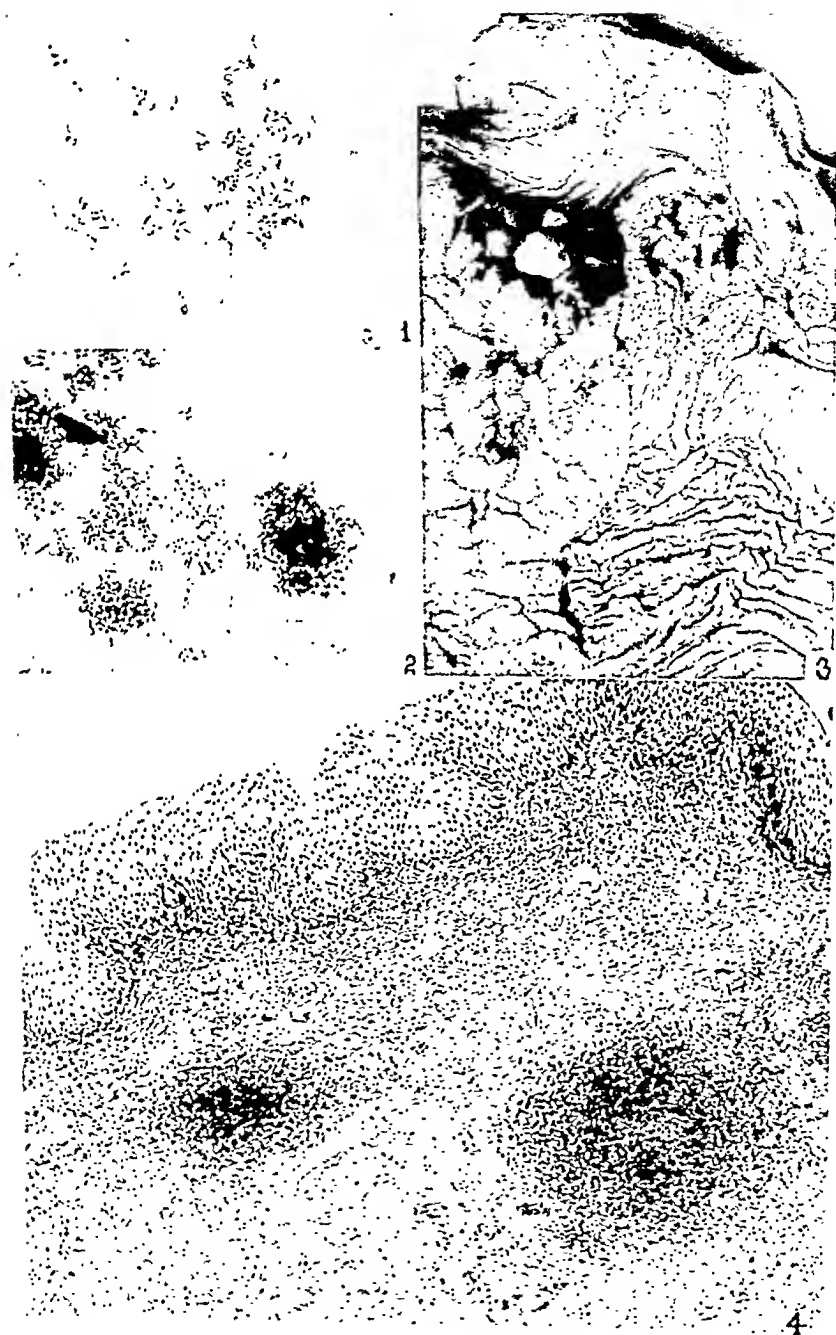
EXPLANATION OF PLATE 1.

FIG. 1. A 48 hour broth culture of the diphtheroid showing one of the characteristic aggregates. Methylene blue. $\times 880$.

FIG. 2. The bacilli in the urinary sediment of Cow 1100, 17 days after inoculation of 3 cc. of broth culture into the bladder. Methylene blue. $\times 880$.

FIG. 3. A portion of the bladder of Cow 1100, 43 days after artificial infection with culture. Note the swollen mucosa studded with dark areas of hemorrhage. About $\frac{2}{3}$ actual size.

FIG. 4. A section of the bladder of Cow 1166 inoculated into the urethra with broth culture 100 days before autopsy. There is a marked infiltration of round cells in the lower portion of the mucosa and a well defined accumulation of round cells in the submucosa. The submucous connective tissue is hyperplastic and a proliferation of blood vessels has occurred. $\times 73$.



IMMUNOLOGICAL STUDIES WITH HERPES VIRUS WITH A CONSIDERATION OF THE HERPES-ENCEPHALITIS PROBLEM.

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I.

Since 1923 one of us has been attempting to transfer virus from human encephalitis cases to animals, using rabbits for most of the experiments and for a few of them monkeys. In the course of this work it has become necessary to make a thorough study of the virus of herpes febrilis, for the obvious reason that this virus has been identified with that of encephalitis lethargica by a great many experienced workers in the past (1). We will deal with our attempts to obtain a virus transmission from man to animals in a later section of this paper. Before approaching this phase of the problem, however, we wish to report briefly upon studies made with the herpes virus itself, and more particularly upon phenomena of immunity which can be experimentally studied with this virus more easily than with most other filterable agents. Moreover, some of the phenomena encountered in this study have, we believe, distinct bearing upon the encephalitis-herpes question.

Our work has been done with three strains of herpes, all of them isolated from vesicles on laboratory workers, some of them carried through several years. Unlike some of the British virus, our own strains have been extremely resistant when preserved in glycerol, one of them recently causing typical death in a rabbit, without material prolongation of the incubation time, after 11½ months in 50 per cent neutral glycerol in the ice box.

Dosage of virus has been a matter to which we have given considerable thought, and in which we have tried to give attention not only

to the actual amounts of brain substance and dilution used, but also to the possibility that the virus might be irregularly distributed throughout the brain. In addition to the method of emulsifying weighed amounts of brain in definite volumes of salt solution, we have followed a method first used in this laboratory by Breinl, in which an entire brain or entire hemisphere was emulsified by grinding in sterile sand, gradual additions of salt solution being made up to 100 cc., and subsequent dilutions made from this. The dilutions in such procedure were calculated from the total brain as a basis. Thus one one-hundred-thousandth of such a dilution would indicate approximately a hundred-thousandth part of the entire brain.

In working with the virus, the interesting observation was made that if prolonged grinding in sand was carried out, and the suspension thus obtained was centrifuged at high speed for an hour or longer until the supernatant fluid was almost clear, rabbits inoculated with such supernatant fluid died often a day or two earlier than those inoculated with the sediment, indicating—to our minds—that in the brain the virus is intracellularly localized and that it is to some extent discharged from the cells in the process of trituration. It is not impossible that the intracellular position of the virus may have some bearing on the irregular results obtained by various observers in experiments upon *in vitro* neutralization by immune sera. Another way of obtaining virus relatively free from cells, which, however, we have not used in these experiments except to determine the possibility, is by tapping the cisterna magna when the animal begins to show symptoms, such spinal fluid being virulent at the time.

Active Immunization.

On this subject little can be added to what is already known, and our results have been consistent in themselves and in harmony with previous reports. The method we have used chiefly has consisted of a preliminary inoculation upon the cornea, followed by intracerebral¹ test doses at varying intervals. Active immunity has regularly followed survival from infection of the eye, appearing not earlier than 2½ weeks after the eye inoculation and lasting up to as long as 6

¹For intracerebral inoculation ether anesthesia was used.

months. Later tests have not so far been made. Active immunity can also be obtained by preliminary skin inoculation as practised by Perdrau, and by intratesticular inoculation.

Neutralization of Herpes Virus with the Sera of Actively Immune Animals.

The literature on this question has been contradictory, some workers denying the neutralizing value of convalescent and immune sera; others, notably Flexner, having no apparent difficulty in such neutralization. Our first three experiments were entirely negative, although we titrated our infecting doses down to five times the minimum fatal amount. In some of these experiments, indeed, the rabbits inoculated with virus-serum mixtures died sooner than the saline controls. This we have now determined is due to a rapid deterioration of the virus when incubated with salt solution, a deteriorating effect prevented in the presence of normal serum.

These negative results agreed in general with those published by Perdrau, but were inconsistent with those of Flexner and others. We therefore continued with them, thinking that probably such differences might be due to fortuitous variations in the sera of individual actively immunized rabbits, depending perhaps upon the method of active immunization, the severity of the herpetic process, or the time factor.

Subsequent experiments were done by a simple technique in which about 50 minimum fatal doses of the virus, that is 0.1 cc. of a 1:10,000 dilution of fresh or glycerolated herpetic brain, were incubated with 1 cc. of either the active or inactivated serum of rabbits that had been immunized by a corneal inoculation and subsequently tested for immunity by an intracerebral one. The mixtures were kept at $37\frac{1}{2}^{\circ}$ from 5 to 6 hours, and amounts of 0.25 cc., representing, thus, something over 10 fatal doses, were intracerebrally injected into normal rabbits. Controls with incubated saline and normal serum suspensions were used.

First Experiment.—The serum of the actively immunized rabbit was obtained 25 days after the second injection. In this experiment the saline control died in 9 days and the rabbit receiving the immune serum-virus mixture gradually lapsed into a lethargic condition from which it recovered in 3 weeks. This

rabbit will be discussed below. No normal serum control was made in this experiment.

Second Experiment.—The serum here used was taken 52 days after the second or intracerebral inoculation of the actively immune animal. The normal serum control died typically on the 4th day, the saline control after 6 days, and the immune serum animal died on the 8th day.

Third Experiment.—The serum used in this case was taken 59 days after the second or intracerebral virus inoculation of the immunized animal. The experiment was somewhat varied from the others in that both activated and inactivated immune and normal serum mixtures were made in order to determine any differences produced by inactivation. The rabbits receiving the mixtures in which immune serum, active and inactive, had been used survived; those in which normal active and inactive serum were used died on the 8th day, while the animal receiving the incubated mixture of virus and saline died after a protracted illness lasting 15 days, and the inactivated virus controls died on the 8th and 11th days, respectively.

From these and similar experiments we may conclude that the serum of rabbits actively immunized by corneal inoculation and subsequently reinjected intracerebrally develop definite protective bodies in their serum which probably do not appear sooner than the 3rd and 4th weeks in sufficient quantity to yield a clean-cut experiment.

Experiments like those above, however, are merely examples of the general type obtained. The results are irregular, thus accounting for discrepancies in the literature, but we do not believe that this need necessarily be accounted for by fluctuations in the potency of the sera themselves.

Irregularities are rather due, we believe, to the fact that in the ordinary technique of using brain material for virus, even when accurately measured dilutions are used, some of the virus is intracellular and some extracellular, and the serum protective bodies, whatever they may be, may therefore be blocked from complete contact with the intracellularly localized fraction. We have discussed this in our introduction, where we mentioned the fact that when virulent brain material is triturated for an hour in sterile sand, taken up in Ringer's solution, and centrifuged for an hour or more, the supernatant fluid, which is clear and relatively cell-free, kills a little more quickly than do equivalent volumes of the sediment.

To some extent the potency of sera, we think, may be enhanced by

what may be spoken of as hyperimmunization. We have treated a number of rabbits after they had developed active immunity with repeated intracerebral injections and large intraperitoneal injections of potent virus, and in this way have obtained sera which exhibited considerably more regularity in the neutralization of herpes virus. Comparative quantitative results cannot yet be made with sufficient accuracy to permit more than a very definite probability in favour of the enhancement of potency by such a method.

Immunization with Brain Extracts.

There has been some discussion in the literature concerning the possibility of the location of the immune substance in the susceptible cells; namely, those of the central nervous system. Here, again, there have been considerable contradictions, Perdrau (2), indeed, finding that the brain extracts of an immunized animal, far from immunizing, increased the virulence of the virus that had been in contact with it. His results in the latter respect may have been due to the salt solution effects that we have mentioned above.

Having determined that the brain suspensions of an immune animal do not retain active virus, we carried out a number of experiments analogous to those carried out with serum in which we incubated active virus with brain extracts of immune animals.

The brain extracts were made by grinding an entire hemisphere with sand for $\frac{1}{2}$ hour, gradually adding 20 cc. of saline. The mixture was shaken for 2 hours, then set in the incubator for an hour, and again shaken for 15 minutes while warm. In some cases it was extracted in the ice chest for longer periods, but in others this was omitted. Finally, the material was centrifugalized at high speed and the supernatant fluid used.

First Experiment.—In this experiment the brain of an immune rabbit was taken out 74 days after the second injection and the rabbit bled at the same time. In other respects the experiment was carried out in a manner similar to that used with the serum tests. In this experiment, curiously enough, there was no protection whatever by the brain extract, and the serum did nothing but prolong the life of the rabbit for 3 days beyond the control, and 2 days beyond the brain extract one, dying on the 8th day.

Second Experiment.—In this experiment, in which the brain was removed from the immune animal 62 days after the second inoculation, the result was approximately the same. Again, the serum protected, prolonging life until the 10th day, 4 days beyond the normal serum control; the brain extract rabbit dying on the 7th day.

These two experiments may therefore be regarded as practically negative.

The following experiment was carried out in connection with those on the protective properties of hyperimmunized rabbit serum.

The brain of a rabbit which had received an eye inoculation on August 31 and subsequently four intracerebral inoculations, the last one on December 21, was taken out 21 days after the last injection. Blood was obtained at the same time. The method of extraction was essentially similar to the one described above. Mixtures were made in which 1 cc. of the fresh brain extract was added, respectively, to 0.15, 0.1, and 0.05 cc. of a 5 per cent fresh virus suspension. A control was made of 1 cc. of the serum of the same rabbit with 0.2 cc. of the virus, and salt solution controls set up with 0.05 cc. of the virus, that is the smallest dose, mixed with the brain. After an incubation of 4 hours, 0.2 cc., respectively, of the mixtures was injected into rabbits. The control died in 9 days; the two rabbits receiving brain extract plus the larger doses of virus died in 5 and 10 days, respectively, while the rabbit receiving the smallest amount of virus plus brain extract, an amount equivalent to that received by the saline control, survived, as did the rabbit receiving the hyperimmune serum plus four times the amount of virus which killed the control and four times the amount of virus which killed one of the brain extract rabbits.

This experiment and another like it seem to prove conclusively that, while the brain extracts of hyperimmunized rabbits may contain a limited amount of protective substance, this is considerably less than that contained in the circulating blood of the same animal.²

*Passive Immunization with Serum by Injecting the Serum Both Intravenously and into the Cisterna Magna the Day before
Injecting the Virus.*

All the experiments done in this direction up to the present time have been completely negative. Neither the intravenous administration of sera known to possess protective action in considerable quantities nor the injection of $\frac{3}{4}$ to 1 cc. of such sera into the cisterna magna the day before intracerebral infection has afforded even the slightest protection to the rabbits so treated.

² Incidentally a considerable number of complement fixations have been done in which filtrates of normal herpetic brains were used as antigen, and attempts have been made at agglutination reactions in virulent filtrates with the serum of immune animals. All of these efforts have remained entirely negative in result.

Are Rabbits Which Survive Neutralized Serum-Virus Mixtures Actively Immune?

In a number of experiments we have subjected rabbits that have survived serum-virus mixtures as above described to subsequent intracerebral inoculation with virus alone. In no case so far have these rabbits shown signs of resistance above the normal.

We report this type of experiment because we believe it adds additional proof to the other observations made that immunity does not follow unless there is an active process at least initiated in the animal during immunization.

Immunization with Phenolized Virus.

In considering the literature on immunity in connection with the filterable viruses, one gains throughout an impression consistent with the observations noted in this paper, that dead virus does not protect, or—in other words—that virus which does not give rise to at least a slight or moderate disease process is not followed by immunity. This, of course, is consistent with the many observations recorded in poliomyelitis, and observers who have studied this are unanimous in stating that monkeys who have failed for one reason or another to “take” may be actively infected in a subsequent experiment. The exception to this rule seems to be the now widely employed method of prophylactic rabies treatment by the Semple method, in which phenolized virus is subcutaneously administered. We have attempted to apply this method to protection against herpes virtually by the same method used by Semple in hydrophobia.

10 per cent suspensions, that is 5 gm. per 50 cc., of fresh herpes virus were made in a 1 per cent carbolic solution. This was incubated for 6 hours, shaken twice while incubating, and left in the refrigerator overnight. This was diluted on the following day with an equal volume of salt solution, making a $\frac{1}{2}$ per cent carbolic suspension.

In attempting protection with phenolized virus made in this way, it seemed to us of the greatest importance to determine whether the carbolic acid killed or merely attenuated the active agent. This determination is incidentally of fundamental importance in appraising the immunological significance of the Semple method in rabies. We

therefore inoculated rabbits intracerebrally with 0.3 cc., respectively, of phenolized virus made as above, 6 hours, 36 hours, 48 hours, and 5 days after the phenol had been added. None of these rabbits, not even the one inoculated with the 6 hour phenolized material, showed any symptoms, even a temperature, and all remained healthy and well. None of them were immunized by the procedure. The only one that died was the 5 day one which died of diarrhea and pneumonia some time after the inoculation.

We can only conclude that the phenol used in the manner indicated by Semple kills the herpes virus.

Two series of three rabbits each were then treated with daily injections of the phenolized virus for 14 days. Test injections with virulent material were made 1, 2, and 3 weeks after the last dose of the phenolized material. In no case was there the slightest protection. Here again, therefore, we are in a position to conclude that no protection whatever is afforded by dead virus.

Believing that possibly the manner of administration might make a difference, we reinoculated intracerebrally some of the rabbits in which the phenolized virus had been administered in the same manner to test whether it was dead or not, and found that these rabbits, likewise, were unimmunized, even though the phenolized material had been given intracerebrally.

II.

The Herpes-Encephalitis Question.—In the course of the last 3 years we have attempted to transfer a virus from human encephalitis to animals with material of some eight cases.³ In at least four of these the pathological examination seemed as reasonably certain in indicating encephalitis lethargica as this is possible, at the present time, by histological examination. In two of the cases where the possibility of poliomyelitis existed, this was ruled out by negative intracerebral monkey inoculation.

Experiments with Rabbits.—A large number of rabbits were used in these experiments, but we do not describe these inoculations in detail

³ We have used spinal fluid from a number of other cases which, however, on later study could not be considered as representing probable encephalitic infection.

because, rigidly analyzed, they must all be regarded as negative. A good many of our rabbits died, and in some of the earlier cases we obtained a few rabbits that died in series up to the fourth generation from the original case. Careful autopsies and cultures in some of these cases revealed other possible causes of death and never have we obtained a typical herpetic syndrome in such animals. While a not inconsiderable number of the rabbits in these experiments died without morphological or cultural evidence of bacterial infection, our inability to carry on a virus in series, the complete absence of anything like an herpetic syndrome, and our later suspicions of possible spontaneous encephalitis justified us in rejecting any suggestions that we had killed any of these animals with a virus emanating from the human encephalitis case.

In our recent cases, in which the rabbits were more closely observed than before, we often obtained a type of languid and almost somnolent condition in many of the inoculated rabbits which was characterized by completely relaxed attitudes in the cages and immobility continuing for hours when the animals were not disturbed. Such animals, however, were always quite alert and normal when roused into activity either mechanically or by the proffer of food, and few of them died, even though observed for long periods. The two or three that did die usually had diarrhea, and transfer of material from their nervous systems did not produce disease in other animals. We believe it likely that the somnolent condition described may represent the clinical picture given by Kling and others which, however, as observed by us, was entirely unconvincing. We may summarize our attempts of directly transferring human encephalitic virus to rabbits with the statement that never, in spite of the use of a large number of animals, have we in any single instance produced anything simulating the herpetic syndrome in such animals, and the fortunate freedom of our experiments from spontaneous rabbit encephalitis has resulted in very few deaths of rabbits which in any manner would have tended to mislead us into thinking that we had made a successful transfer.

Attempt to Repeat Perdrau's Method of Repeated Inoculations.—Perdrau has recently described a procedure with which he reports successes in starting an herpetiform virus in rabbits by repeated inoculations of glycerolated encephalitic brain given on alternate days,

three inoculations to each rabbit, alternately on the skin, into the brain, and again upon the skin. He has varied this in a number of different ways. He also reports success with intracerebral inoculations of an encephalitis virus which had been preserved in glycerol for 43 days in cases in which the fresh, unglycerolated virus gave negative results. He furthermore succeeded by inoculating, together with the human encephalitic brain material, suspensions of the brain of herpes immune rabbits in which, by his own control tests as well as in repetitions of our own, no surviving herpes virus can be detected.

We tried Perdrau's method with material from three cases in two of which there seemed to be no possible question as to clinical or pathological diagnosis, and in one of these cases we persisted with the repeated inoculation technique quite beyond Perdrau's original report, administering in some cases as many as five injections. In no case did we either succeed in producing an herpeticiform syndrome nor did we obtain any other type of disease in the rabbits which might have been attributed to a transmissible virus. Again, however, we noticed the prolonged somnolence, temporary loss of appetite, and that slight departure from normal which has been described above. In no case, however, did these rabbits develop the temperature changes which we have found so uniform in successful herpes inoculations.

In view of the failure of direct repetition of Perdrau's experiments, and considering the results with partial immunization of herpetic rabbits which will be recorded below, we thought it entirely logical to subject the animals treated by repeated injection of the encephalitic virus to subsequent herpes inoculation, with the idea of determining whether or not the development of a partial immunity would indicate the presence of perhaps an attenuated herpes-like virus in the encephalitis brains.

Altogether fourteen rabbits treated by the Perdrau method or some modification of it, all of them having received at least four consecutive inoculations of encephalitic material, were reinoculated with measured doses of herpes virus at periods sufficiently long after the last inoculation to warrant the expectation of some immunity. All these animals died at periods sufficiently close to those at which the controls succumbed to exclude the possibility of their having been even partially immunized by the injection of the encephalitic brain.

Partial Immunization.—Among the most interesting of the results of our experiments are those in which, either purposefully or by accident, the partial immunization of the rabbits to herpes virus was produced. These observations were begun a year ago in experiments carried out in this laboratory by Dr. Breinl.⁴ They have since been extended in various ways. They are similar in result to some of those reported by Perdrau. Partial immunization was obtained either by the injection of sublethal doses of herpes virus, by which we mean the injection of 0.1 cc. of a 1:50,000 to a 1:200,000 dilution of a brain emulsion fatal in amounts of 1:10,000, or they were obtained by immunization with serum-virus mixtures. In one case the effects to be described were observed after the first inoculation, when a virus was injected which had been incubated with salt solution and thus attenuated, as described in a preceding section. It is not always possible to produce such rabbits at will, but when successful, the animals, after a slight preliminary temperature, develop gradual drowsiness and weakness at about the time that the controls die, and may go to sleep, remaining in this comatose condition anywhere from 4 to 8 days before death. Animals similar to these have been described by Perdrau. The brains of such rabbits are virulent.

One rabbit in this series is particularly interesting. This was a rabbit of about 2500 gm. which was injected on October 21 with 0.1 cc. of a 1:10,000 herpes virus obtained from the fourth brain passage of this particular material after 20 days in glycerol. The virus had been incubated for 5 hours at 37°C. before injection, which—as other experiments show—materially attenuates the virus. The animal at first showed no symptoms, but 3 weeks after inoculation it was found in a lethargic state, sleeping most of the time. Slight stimulation rapidly removed the drowsiness into which the animal at once relapsed as soon as stimulation was interrupted. The animal remained in this condition for 3 weeks, when a gradual recovery to normal occurred. During this entire course there was no fever, and careful examination elicited neither tremors nor paralysis. Muscular weakness seemed to be associated with the drowsiness, even when temporarily roused. On January 25, 1926, the animal was tested for immunity with 0.15 cc. of a 5 per cent virus suspension of a potent virus. 5 days later it developed a flaccid paralysis of the hind legs. There was no fever, but there was typical

⁴It was unfortunate that Dr. Breinl had to leave at a time when this work was started. Experiments referred to in the text were subsequently extended from observations made while he was still in this laboratory.

grinding of the teeth. A control done with the same virus at the same time died on the 30th with typical symptoms. On January 31 the condition took the form of a gradually ascending paralysis, the animal lying on its side and still moving its front legs, the head slightly bent to one side, as with a spasm. This condition continued throughout February 1. Death occurred on February 2, the entire clinical picture from beginning to end being as parallel as possible in clinical story with Case 9 of our human encephalitis series.

These experiments were considered of great importance because, in spite of all of the preceding negative evidence, the clinical picture in these cases is so strikingly similar to the conditions observed in diseased human beings that they rather open the possibility that the encephalitis and herpes virus may still be related, but that in passing through the nervous system of man and remaining there for some time, the herpes virus may have become so attenuated for rabbits that it can no longer be started in these animals unless exceptionally fortunate cases are encountered. Such exceptional circumstances might explain the ease with which earlier investigators obtained rabbit transmission, as contrasted with the negative results of Flexner and Amoss' (3) experiments and our own. Moreover, it is not at all impossible that the positive findings of herpes virus in the central nervous systems of human beings not suffering from encephalitis, as well as the innocuousness of encephalitis virus administered to men, as in Busacca and Bastai's (4) experiments, might be explained by an immunity to the virus existing in many people. For it is more than probable that human beings may develop a considerable resistance against the deeper penetration of a virus so ubiquitous in our environment and so frequently causing lesions of minor importance on the skin and mucous membranes. We mention these things because it is necessary in a subject as important as this one to consider all points of view as broadly as possible. Moreover, the extraordinary attenuation of fixed rabies virus for man furnishes a closely pertinent analogy.

SUMMARY.

In the preceding experiments observations have been reported upon the nature of herpes virus which confirm the suspicion that the virus is intracellularly located in the infected nervous system.

In regard to the immunological conditions existing in this disease, our experiments have reaffirmed that herpes virus can be neutralized with the serum of actively immunized animals and have offered an explanation for the irregularity of the results of others, as well as our own.

It has been found that brain extracts possess some virus-neutralizing power, but considerably less than the serum of the corresponding animals.

Attempts at passive immunization with neutralizing serum were uniformly negative, even when the serum was introduced into the *cisterna magna* 12 to 24 hours before infection with the virus.

It has been shown that active immunity can be attained only when some degree of reaction to the living virus has occurred. Rabbits which survived neutralized serum-virus mixtures did not acquire immunity nor did those treated with virus phenolized to the extent of actual destruction. This point suggests a reinvestigation of the Semple method of rabies immunization.

In so far as our studies touched upon the herpes-encephalitis problem we have uniformly failed in attempts to transfer herpes virus directly from man to rabbits. These results are in contradiction to those of most of the earlier workers, but in keeping with the recent reports of Flexner and Amoss.

Attempts to overcome the difficulty of transfer by the recently published technique of Perdrau were unsuccessful. Furthermore, animals repeatedly treated with human encephalitis material, either fresh or glycerolated, as practised in the Perdrau method, failed to acquire the slightest degree of immunity to subsequent herpes inoculation.

By the inoculation of very small doses or by infection of partially immunized rabbits, as described above, we have succeeded in modifying the characteristic herpetic syndrome in rabbits in a manner which simulates many of the clinical features of human encephalitis.

Our own experience forces the conclusion that no valid proof exists upon which can be based an assertion concerning the identity of the virus of herpes with that of encephalitis lethargica. Either the two viruses are entirely unrelated, or else prolonged sojourn in the central nervous system of man attenuates the virus for rabbits to an extent

analogous to that in which rabies virus is attenuated for man by passage through rabbits. The isolated successes of Levaditi and of Doerr and their assistants might thus be regarded as fortunate exceptions in which material incompletely attenuated had been at their disposal.

We suggest this point of view as an alternative working hypothesis largely because the results we are reporting, as well as those of Flexner and Amoss, are in flat contradiction to the reported successes of earlier workers and the more recent experiments of Perdrau. The experiments of the latter, as described, cannot be explained by the occasional existence of spontaneous encephalitis in his rabbits, nor by the assumption that a herpes virus fortuitously coexisted with that of lethargic encephalitis in his material, inasmuch as this material alone at first injection or in the unglycerolated state failed to infect. It is also possible to conceive that human beings may, by repeated skin infections, attain a not inconsiderable partial immunity to herpes virus, which would explain the nature of the clinical course (as in our partial immunity rabbits) as well as the innocuousness of direct injections of herpetic virus into man, as reported by Bastai and Busacca, and the finding of herpes virus in human beings not suffering from lethargic encephalitis.

These suggestions are discussed in order to give this important problem the broadest possible consideration.

For the time being, however, such reasoning cannot be taken as more than a logical possibility impressed upon us by our partial immunization experiments.

All other experimental evidence obtained by direct inoculations with the limited material at our disposal tends to render identity of the two varieties of viruses unlikely.

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THE SKIN RESPONSE OF RABBITS TO NON-HEMOLYTIC STREPTOCOCCI.

I. DESCRIPTION OF A SECONDARY REACTION OCCURRING LOCALLY AFTER INTRADERMAL INOCULATION.

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PLATE 2.

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INTRODUCTION.

The present paper contains a description of a remarkable and unexpected series of events which followed the inoculation of non-hemolytic streptococci into the skins of rabbits. Following the studies of Rivers (1) on the skin lesions produced in rabbits by the intradermal inoculation of hemolytic streptococci, we applied similar methods with the non-hemolytic streptococci, but immediately encountered a phenomenon quite different from anything described by previous observers with any type of bacteria. In brief, it was found that the intracutaneous inoculation of rabbits with certain non-hemolytic streptococci produced a raised lesion, often 20 to 30 mm. across, which reached its maximum size in 24 to 48 hours and then began to retrogress; about the 8th or 9th day, however, an increase in the size and redness of the lesion occurred in many animals. This period of increased activity lasted for 2 or 3 days; then the lesion gradually disappeared. This phenomenon has been designated the "secondary reaction;" it seemed to us worthy of detailed study.

EXPERIMENTAL.

Technique.—Adult rabbits weighing from 1200 to 2500 gm. were used. The hair on their flanks was clipped short with scissors and then completely removed with a solution of sodium sulfide consisting of 35 gm. to 60 cc. of water. As a rule this depilation was carried out several days before the animals were inocu-

lated. Parallel experiments showed that the cutaneous responses to inoculation were similar whether the skin was prepared by this method of depilation or by shaving with a razor. 18 hour cultures of bacteria in 0.5 per cent rabbit blood broth were used for inoculation; plain broth cultures gave comparable results; but since the streptococcus most studied, Strain V92, grew very poorly in plain broth, it was thought best to use rabbit blood broth throughout. In most experiments the cultures were made in 50 cc. of broth; the growth was centrifuged and the sedimented organisms made up to 1 cc. with normal salt solution. Thus 0.1 cc., the quantity usually inoculated, contained the organisms from 5 cc. of the original culture. Inoculations were made intradermally on the dorsal portion of the rabbits' flanks. The two diameters of the lesions were measured with calipers daily by the same observer. The size of any lesion is represented in the charts by the sum of these two diameters (see Chart 1).

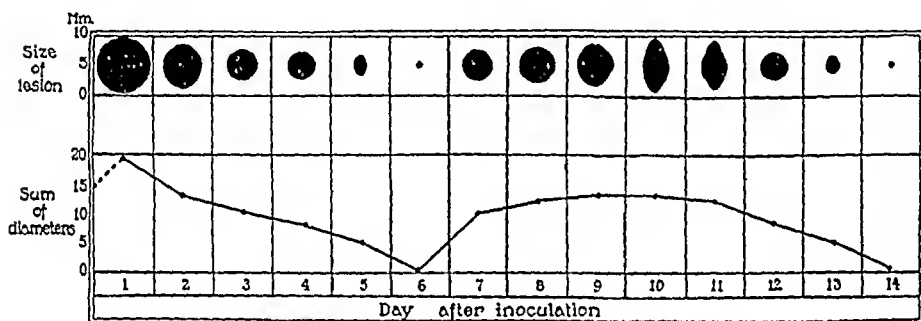


CHART 1. Showing results of a small inoculation of green streptococcus (V92) in a rabbit's skin. Above, the actual size of the lesion from day to day is represented. Below, is plotted a curve of the variations in size, when the sum of the two diameters of the lesion is taken as the index.

Early in the work most of the inoculations were with one strain of *Streptococcus viridans* (V92) which had been isolated from a subcutaneous fibroid nodule of a patient with rheumatic fever.

Primary Reaction.—When the deposit from 5 cc. of a culture of this strain was inoculated as described, a lesion of fairly uniform character resulted; after 24 hours its diameters were in the neighborhood of 30 by 20 mm.; it was raised 3 or 4 mm. above the surface of the surrounding skin; it was red, especially towards the middle; and near the dorsal end there was usually a small yellow or greenish center. Surrounding this central nodule was a definite zone of edema and erythema which fused gradually with the surrounding skin. As a rule the size of the

lesion ceased to progress after 36 to 48 hours. A rapid diminution in size then began; the surrounding edema decreased rapidly and the central nodule more gradually. This nodule often became covered by a scab after 2 or 3 days, usually without much purulent discharge. Not infrequently, however, there was neither scab formation nor breaking down of the skin over the lesion. Where no secondary reaction occurred the nodule continued to retrogress; commonly it was no longer palpable after 3 weeks.

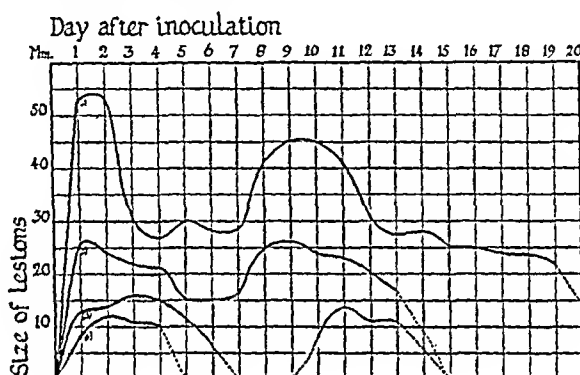


CHART 2. Showing types of curves with secondary reactions when a rabbit was inoculated intradermally at the same time in four different places with different sized doses of *Streptococcus* V92, as follows:

Curve (1) sediment from 5 cc. of blood broth culture.

" (2) 0.1 cc. of blood broth culture.

" (3) 0.01 " " " " "

" (4) 0.001 " " " " "

Secondary Reaction.—In more than half of the rabbits, however, a change set in about the 8th or 9th day (see Fig. 1). The whole lesion, which had become fairly pale, took on once more a bright pink color and began to enlarge with an average increase of 3 or 4 mm. in each diameter; but, at times, the increase took place in only one direction, usually ventralward, but sometimes dorsalward, or laterally. Frequently small outlying red satellite nodules appeared; these sometimes fused later with the main lesion. Occasionally a lesion at the time of the secondary reaction attained a size greater than it had immediately following

inoculation. Doses of 0.1, 0.01, and 0.001 cc. of streptococci produced proportionately smaller primary lesions. With 0.1 cc. doses the lesion never disappeared completely before the onset of the secondary reaction, and the secondary reaction usually occurred in the same animal simultaneously with that seen in lesions produced by larger doses. With 0.01 cc. doses, however, the primary lesions usually disappeared after the 4th to 5th day, but in the rabbits having a secondary reaction there was a recurrence of an erythematous papule slightly later than the time of the maximum secondary reactions seen in larger lesions. No secondary reaction has been observed at the site of inoculation of 0.001 cc. (see Chart 2). No lesion ever broke down at the time of the secondary reaction. The secondary reaction lasted from 1 to 3 days and then subsided. The total duration of a lesion following 5 cc. or 0.1 cc. inoculations was from 1 to 2 weeks longer in those that showed a secondary reaction compared with those that did not. A definite third period of activity was not observed.

The secondary reaction did not occur in all rabbits inoculated with *Streptococcus* V92 (see Fig. 2). Of 151 injected with living cultures of this organism, 79 showed a definite secondary reaction and 54 failed to show it; in 18 there was a very slight increase of only 1.5 or 2 mm. in each diameter; hence, it was doubtful whether or not a true secondary reaction occurred; reactions of this order are classed as "doubtful" in the tables. Increases in the size of a lesion of 1 mm. or less in each diameter are recorded as negative. According to whether the "doubtful" group is, or is not, counted as positive, either 52 or 64 per cent of the rabbits may be said to have shown secondary reactions following inoculations of *Streptococcus* V92. The occurrence or non-occurrence of a secondary reaction appeared to be independent of the age, sex, and color of the rabbits. Since secondary reactions did not occur in 100 per cent of normal rabbits it was found necessary to use three or more animals for every experiment and for every control in order, as far as possible, to avoid false conclusions.

While the height of the secondary reaction was usually on the 8th or 9th day following inoculation, it also occurred not uncommonly on the 7th or 10th day, rarely earlier or later than this. The actual figures for the day of maximal secondary reaction in 117 animals are as follows:

4th day.....	2 rabbits.	10th day.....	23 rabbits.
5th "	2 "	11th "	9 "
6th "	5 "	12th "	6 "
7th "	11 "	13th "	4 "
8th "	23 "	14th "	1 "
9th "	30 "	16th "	1 "
= 117 rabbits.			

The lesions produced were studied both bacteriologically and histologically. On several occasions we inoculated rabbits intradermally in six or eight places and subsequently excised a lesion every day under ether anesthesia. These lesions were studied by staining direct films, by making cultures, and by histological methods.

Bacteriological Studies.—The films, stained by Gram's method, showed many polymorphonuclear leucocytes from the beginning and, after the 6th day, some macrophages as well. Streptococci were seen in large numbers at first but became progressively fewer, until they were usually no longer visible after the 6th or 8th day; on two occasions, however, they were still seen on the 10th and 12th days respectively. They were often found phagocyted either by polymorphonuclear leucocytes or by macrophages; it must be supposed that they were usually dead, as they were not infrequently seen in films when they could no longer be recovered in cultures. In making cultures the excised tissues were pinned out on cork with the epidermal surface downwards, and a portion of the lesion was removed under aseptic conditions from the exposed surface without cutting through the epidermis. We thus hoped to avoid contamination, but, even with this technique, a few colonies of contaminants, especially staphylococci, appeared from time to time in the cultures. The excised tissue was finely divided with scissors, ground with sterile sand and normal salt solution with a pestle and mortar, and inoculated both into broth and into poured blood agar or dextrose blood agar plates. Streptococci always grew plentifully from 1 day old lesions, less abundantly from those 2 days old, and occasionally none were obtained from lesions 3 days old. On three occasions, however, a growth was obtained 7 days after the rabbit was inoculated, and once after 9 days. There was never any evidence of an increase in the number of streptococci at the time of the secondary reaction; in fact, streptococci were never recovered from lesions at the time of a secondary reaction.

Histological Study.—The tissues were fixed in Zenker solution containing acetic acid and stained with eosin and methylene blue and with Gram's stain. The histological changes were as follows:

24 hours after inoculation the lesion consisted of a mass of polymorphonuclear leucocytes and bacteria forming a small abscess with ill defined walls. Edema and hyperemia were present in the surrounding tissues. A number of mononuclear cells, apparently formed as a result of vascular endothelial proliferation, were commonly seen around the small blood vessels in the deepest part of the lesion. During the next 3 days, granulation tissue gradually formed outside the mass of polymorphonuclears, until by the 5th or 6th day the central abscess was fairly well shut off by a wall of young connective tissue. This wall was more marked in lesions that followed the larger doses, and less in the smaller lesions. Rather regularly about the 6th day numbers of macrophages appeared at the periphery of the mass of disintegrating polymorphonuclears and apparently acted as scavengers. In lesions removed from rabbits which showed no secondary reactions in duplicate areas not excised, there appeared on the 5th and 6th days collections of small round cells—both lymphocytes and plasma cells—about many of the blood vessels a short distance from the abscess wall. Similar collections of cells were also present in the young connective tissue making up the abscess wall. These collections of small round cells became more distinct for 2 or 3 days, then gradually decreased as the fibrous tissue making up the wall became more dense. In the tissues of animals which showed a secondary reaction similar collections of small round cells were not observed until the end of the secondary reaction.

At the time of the secondary reaction a distinct type of histological response was noted.

In the small blood vessels, especially in the arterioles just outside of the granulation tissue there was a marked swelling of the endothelial cells which assumed a cuboidal shape and gave the vessel the appearance of an epithelial lined gland duct. Spaces around some of the vessels were also packed with large polygonal endothelioid cells having a basophilic protoplasm; in other places masses of similar cells were seen not as intimately associated with blood vessels. Numerous isolated swollen connective tissue cells were also present in the subcutaneous tissue, corium, and between the muscle fibers more distant from the abscess wall. In these more remote zones the connective tissue fibers were more widely separated than normal, probably the result of edema.

In satellite nodules excised at the height of the secondary reaction there was a central area made up of a mixture of pycnotic and fragmented nuclei, polymorphonuclear and mononuclear cells, and amorphous granules. This was surrounded by masses of polygonal endothelioid cells, and by blood vessels showing marked endothelial proliferation. A moderate number of polymorphonuclears were present in this zone. In secondary reaction tissue a few days older the

endothelioid reaction became less marked, masses of small round cells appeared in the perivascular regions, and the tissues gradually assumed the appearance seen in the simple lesion which did not show secondary reaction.

Bacteria Giving Rise to a Secondary Reaction.—It was naturally of interest to know just what bacteria could produce the secondary reaction described. Was this property peculiar to the strain of streptococci used, was it common to all green streptococci, or would it follow inoculation of other microorganisms? Ten other strains of *Streptococcus viridans*, all isolated from subcutaneous nodules, blood, or hearts of patients with rheumatic fever, were accordingly studied. Five of these gave rise to well marked secondary reactions in 50 per cent or more of animals tested. No secondary reaction, or at most a doubtful one, followed inoculation of the other five strains; it is true that they were not tested on large series of rabbits. Details are given in Table I. At first we failed to induce this reaction with *Streptococcus viridans* isolated from sources other than rheumatic fever; two strains from normal throats gave no definite secondary reaction; three strains from the blood of patients with *Streptococcus viridans* endocarditis gave negative results; a fourth strain (T4), however, from a patient with subacute bacterial endocarditis induced two definite and two doubtful secondary reactions. In this patient the endocarditis was grafted upon a congenital heart lesion, and there was no evidence of past or present rheumatic fever.

A few other streptococci were studied. One (T106), which was indifferent to blood and came from the urine of a patient with nephritis, caused secondary reactions in all three rabbits tested, even though there were very trifling primary reactions. An anomalous streptococcus (T27B), which was isolated from a subcutaneous rheumatic fibroid nodule caused hemolysis on rabbit blood agar plates but not in liquid media containing blood and fermented mannitol but not lactose; this organism produced a secondary reaction in two out of four rabbits.

Because of the close relationship between *Streptococcus viridans* and the pneumococcus it was of special interest to test the capacity of the latter to induce secondary reactions. Intracutaneous inoculations of rabbits with living virulent pneumococci produced large necrotic lesions and caused the animals to die in 3 to 5 days. It was, therefore, necessary to use heat-killed virulent pneumococci or aviru-

TABLE I.
The Incidence of Secondary Reactions in Rabbits Injected Intradermally with Various Bacteria and Bacterial Products.

Type of bacteria.	Strain.	Source.	No. of rabbits.			
			Inoculated.	With definite secondary reaction.	With doubtful secondary reaction.	Without secondary reaction.
Living:						
Green streptococcus.....	V92	Rheumatic fever.	151	79	18	54
" "	V110A	"	8	4	0	4
" "	V110B	"	4	4	0	0
" "	V119	"	2	2	0	0
" "	38D	"	8	3	1	4
" "	A49	"	2	1	0	1
" "	A135	"	2	0	1	1
" "	W72	"	3	0	0	3
" "	T27h	"	5	0	1	4
" "	T20a	"	2	0	1	1
" "	T20b	"	2	0	0	2
" "	A148	Bacterial endocarditis.	2	0	0	2
" "	W67	"	2	0	0	2
" "	T57	"	3	0	0	3
" "	T4	"	9	2	2	5
" "	T51	Normal throat.	4	0	0	4
" "	T52	"	4	0	1	3
Indifferent streptococcus.....	T106	Nephritis.	3	3	0	0
Avirulent pneumococcus.....	Type IR	—	13	2	0	11
Hemolytic streptococcus:						
Anomalous "	T27b	Rheumatic fever.	4	2	0	2
Bovine "	Manhattan.	—	3	0	0	3

Human streptococcus.....	S3	Bronchopneumonia.	6	0	0	6
" ".....	NY5	Scarlet fever.	1	0	0	1
" ".....	T43	Rheumatic "	3	0	0	3
" ".....	S23	Lobar pneumonia.	3	0	0	3
" ".....	T10	Rheumatic fever.	3	0	0	3
" ".....	T40	" "	3	0	0	3
Staphylococcus.....	Various.	—	21	1*	1	19
<i>Micrococcus catarrhalis</i>	—	Normal throat.	4	0	0	4
<i>B. coli</i>	—	" stool.	6	0	0	6
Heat-killed:						
Green streptococcus.....	V92	—	17	6	0	11
Virulent pneumococcus.....	Type IS	—	9	1	1	7
Avirulent ".....	" IR	—	6	0	0	6
Hemolytic streptococcus.....	S3, S23	—	9	0	0	9
Products of green streptococci:						
Nucleoprotein.....	T4, V92	—	9	0	0	9
Soluble substance.....	T4	—	4	0	0	4
Culture filtrate.....	V92	—	10	0	0	10

* See text (page 44).

lent living forms.¹ In thirteen animals tested with avirulent living cultures (R form) there were two secondary reactions, on the 11th and 14th days respectively; six tested with similar cultures heat-killed (56°C. for 1½ hours) showed no secondary reactions. Virulent Type I (S form) heat-killed in a similar manner induced one definite and one doubtful secondary reaction in a total of nine rabbits tested; these reactions occurred early—on the 5th day. It seems, therefore, that the pneumococcus has the capacity of inducing secondary reactions, but this is less marked than in some strains of green streptococci.

Bacteria Giving Rise to No Secondary Reaction.—In striking contrast to the other members of the streptococcus group, inoculation of typical hemolytic streptococci uniformly failed to be followed by a secondary reaction. Six strains, a scarlet fever streptococcus included, were tested on nineteen rabbits. It may be mentioned that two out of three of these died within 2 days of receiving the scarlet fever streptococcus—a result which never followed the injection of any other streptococcal strain. It must be borne in mind that the inflammation resulting from hemolytic streptococcus injection was considerably more extensive than that caused by green streptococcus; this fact in itself may have had some influence on the subsequent course of events. One strain of bovine hemolytic streptococci was tested on three rabbits without causing any secondary reaction.

With one exception, injections of staphylococci never led to the production of a secondary reaction. Four strains were tested on twenty-one rabbits. The exception is of some interest in that it occurred in a rabbit which was inoculated at the same time with green streptococcus (V92); when the streptococcus lesions increased in size the staphylococcus lesions increased simultaneously. The experiment was repeated without reproducing this result; hence its significance is not clear. *Micrococcus catarrhalis* failed to induce a secondary reaction in any of four rabbits; *Bacillus coli* did not do so in any of six rabbits tested.

Results of Second Inoculations.—It naturally was of interest to determine whether or not this secondary reaction would appear re-

¹ For the cultures of avirulent pneumococci we are indebted to Dr. H. A. Reimann who produced these "rough" forms from virulent "smooth" forms by various manipulations (see Reimann, H. A., *J. Exp. Med.*, 1925, xli, 587).

peatedly in one animal. A rabbit, having received an intradermal inoculation of 5 cc. green streptococci and a subsequent similar inoculation of the same strain within a period of 9 weeks, showed primary reactions of approximately the same intensity following both injections, but within this period never gave a secondary reaction after the second injection. This was equally true whether the second inoculation was on the same or the opposite side of the animal. Whenever previously inoculated rabbits were retested with Strain V92, equal numbers of normal animals were used as controls in order to be certain

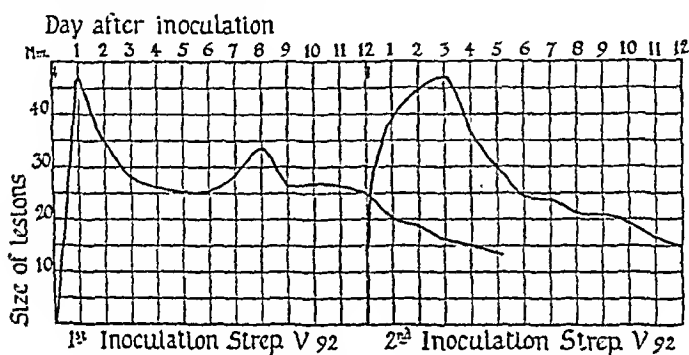


CHART 3. Showing that a rabbit which gave a secondary reaction after first inoculation with Strain V92 failed to give a secondary reaction after the second inoculation with the same strain. Arrows indicate times of inoculation.

that the strain still regularly induced a secondary reaction. Thus twenty rabbits which had received an intradermal inoculation of Strain V92 from 1 to 9 weeks previously all failed to show a secondary reaction following a later injection (see Chart 3). The same result was obtained with two rabbits inoculated with Strain V110A and two with Strain V110B; all four had shown well marked secondary reactions after their first inoculations. This inhibiting effect was not specific; a previous injection with any green streptococcus, whether or not it itself produced a secondary reaction, sufficed to prevent a reaction occurring after a later injection with Strain V92 (see Chart 4). Thus Table II shows that this inhibiting effect occurred after preliminary treatment with Strains T51, T52, and T27h. Six intravenously im-

munized rabbits also failed regularly to show any secondary reaction. The duration of this altered reactivity of the animal—we hesitate to call it immunity—was not thoroughly studied. Rabbits do, however, regain their ability to show secondary reactions after a period of at least 9 weeks, for among thirteen animals inoculated more than 9 weeks after their first injection six showed no secondary reaction, two a doubtful, and five a definite reaction.

Typical hemolytic streptococci, it will be recalled, never gave rise to secondary reactions; nevertheless a previous injection with living cul-

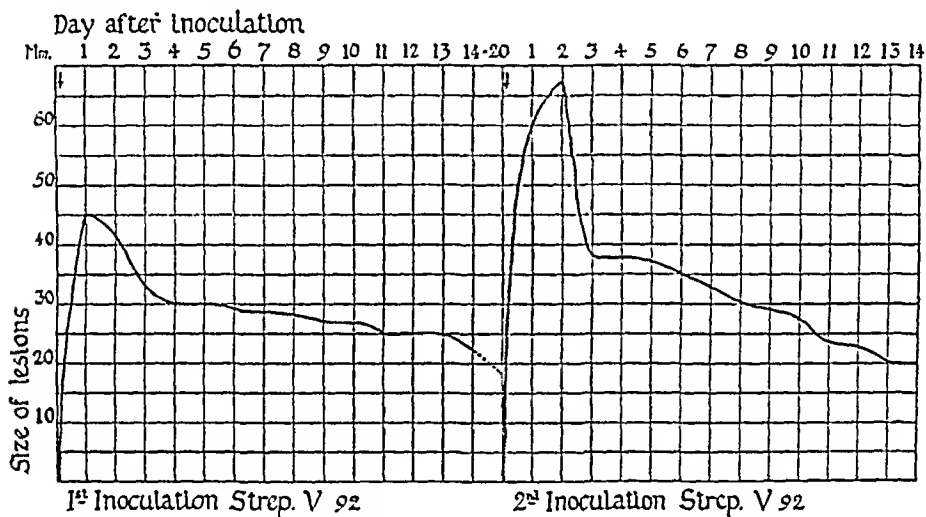


CHART 4. Showing that a rabbit which did not give a secondary reaction after first inoculation with Strain V92 also failed to give a secondary reaction after the second inoculation with the same strain. The inoculation with any streptococcus has a similar protecting effect.

tures of these organisms regularly inhibited the development of a secondary reaction when a green streptococcus, such as Strain V92, was inoculated into an animal a few weeks later. This inhibition was seen four times after intradermal inoculations of hemolytic streptococci and six times after the rabbits had been inoculated with hemolytic streptococci into subcutaneous agar foci. In nine animals which received a preliminary injection of heat-killed hemolytic streptococci there was one definite and one doubtful reaction following inoculation with Strain V92.

Preliminary inoculation with pneumococci, on the other hand, was less effectual in preventing secondary reactions after a later injection with green streptococci. Thus among a total of twenty-eight rabbits first inoculated with pneumococci, six gave definite and two doubtful reactions after later inoculations with *Streptococcus* V92. Similar results were obtained whether the pneumococci were living or heat-killed Type IR or heat-killed Type IS.

Inoculations with microorganisms not belonging to the streptococcus group, on the other hand, appeared to be unable to prevent the second-

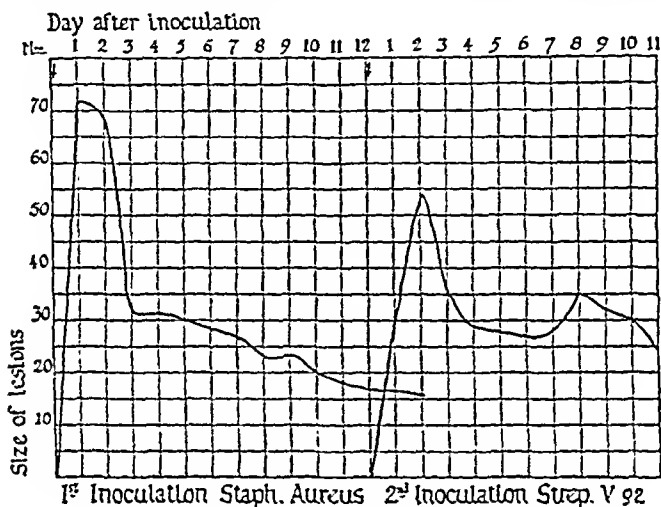


CHART 5. Showing that a preliminary inoculation with *Staphylococcus aureus* does not prevent a secondary reaction after inoculation with *Streptococcus* V92.

ary reaction (see Chart 5). Of nine rabbits injected first with staphylococci and within 9 weeks with *Streptococcus* V92, four showed good secondary reactions; similar results are recorded in Table II in animals previously treated with *Bacillus coli* and with *Micrococcus catarrhalis*.

Results of Inoculations with Killed Bacteria.—The results of cultures from the lesions indicated that the secondary reaction was not due to an increased activity on the part of the microorganisms injected. Cultures of green streptococci (V92) were killed by heating at 56°C. for an hour or by 1 per cent phenol and then shown to be sterile; when

TABLE II.

Effect of Various Bacteria and Bacterial Products in Preventing the Appearance of a Secondary Reaction When Green Streptococci Were Subsequently Injected.

First inoculation.			Subsequent inoculation.			
Bacteria.	Strain.	Production of secondary reaction.	Strain *	No. of rabbits.		
				With definite secondary reaction.	With doubtful secondary reaction.	Without secondary reaction.
Living:						
Green streptococcus.....	V92	+	V92	0	0	20
“ “	Intravenous V92	—	V92	0	0	6
“ “	V110A	+	V110A	0	0	2
“ “	V110B	+	V110B	0	0	2
“ “	T27h	0	V92	0	1	2
“ “	T4	+	V92	0	0	3
“ “	T51	0	V92	0	0	2
“ “	T52	0	V92	0	0	2
“ “	V92	+	†V92	4	2	5
“ “	V110B	+	†V110B	1	0	1
Pneumococcus avirulent.....	Type IR	+	V92	1	1	11
Hemolytic streptococcus.....	S3	0	V92	0	0	3
“ “	NY5	0	V92	0	0	1
“ “	T10 agar foci.	—	V92	0	0	6
Staphylococcus.....	—	0	V92	4	0	5
Micrococcus catarrhalis.....	—	0	V92	1	1	1
B. coli.....	—	0	V92	3	0	3
Heat-killed:						
Green streptococcus.....	V92	+	V92	2	0	10
Virulent pneumococcus.....	Type IS	+	V92	3	1	5
Avirulent “	“ IR	0	V92	2	0	4
Hemolytic streptococcus.....	S3	0	V92	1	1	4
“ “	S23	0	V92	0	0	3
Products of green streptococci:						
Nucleoprotein.....	T4, V92	0	V92	2	0	3
Culture filtrate.....	V92	0	V92	2	1	8

*All subsequent inoculations were within 9 weeks of the first inoculation unless indicated with the sign†.

inoculated as usual into rabbits, they gave rise to lesions similar to those caused by live cultures and not very much smaller. Of seventeen rabbits so inoculated, six showed secondary reactions as marked as were usually seen following live cultures (see Table I). These killed cultures were demonstrated also to be almost as effective as live ones in preventing secondary reactions from following a later dose of live organisms. This prevention was complete in ten out of twelve animals; and in the other two a small secondary reaction occurred. Nine rabbits were injected with hemolytic streptococci killed at 56°C. without showing a secondary reaction; but upon reinjection with *Streptococcus* V92 one showed a definite and one a doubtful reaction.

Since the whole group of streptococci was apparently concerned with the phenomenon under study and since Lancefield (2) had shown that the nucleoproteins obtainable from various members of the group are antigenically related, we studied lesions produced by injection of some nucleoproteins derived from two strains, T4 and V92, which she kindly gave us. These products produced definite lesions in the rabbits' skins, but induced no secondary reaction in any of nine rabbits; nor did nucleoprotein injections prevent the appearance of a secondary reaction in response to a subsequent inoculation of live streptococci after an interval of 12 or 15 days. No secondary reaction was ever seen to follow the injection into rabbits' skins of soluble precipitable substance (residue antigen) prepared by Dr. Lancefield from a green streptococcus (T4), nor did it follow the injection of broth filtrates of streptococcus cultures. The subcutaneous or intradermal injection of these filtrates, moreover, did not prevent the occurrence of secondary reactions when the rabbits were inoculated intradermally with living cultures of *Streptococcus* V92 after 5 to 17 days.

Results in Guinea Pigs.—Twelve guinea pigs were inoculated into the skin of the shaved abdomen with strains of green streptococci (V92, V110A, V110B) known to produce secondary reactions in rabbits: in no guinea pig was a definite secondary reaction observed within a period of 2 weeks; they were not observed longer. Tests have not as yet been made on other laboratory animals.

DISCUSSION.

A phenomenon referred to as a "secondary reaction" in rabbits' skins has been described; it can be regularly produced in a certain percentage of rabbits; its interpretation offers difficulties which have not as yet been surmounted. It is felt, however, that some of the possibilities which immediately suggest themselves can be definitely excluded. Our natural thought at first was that the bacteria originally injected showed an increased activity at the time of the secondary reaction, or that the defensive mechanism of the host had temporarily broken down. The first idea at least was negatived by our failure to recover streptococci from the lesions at the time of the secondary reaction and by the appearance of the reaction after the introduction of killed bacteria. It does not seem probable, moreover, that the phenomenon is due to secondary infection, because frequently the lesions did not ulcerate at any time, and they commonly failed to yield any growth on culture. The staphylococci occasionally encountered were never numerous. If these possibilities are excluded, there are three possible sources for a hypothetical substance which might excite the secondary reaction. This substance may be: (1) a product of the streptococci; (2) a product of the animal host; (3) a product of the interaction of the streptococci and animal host.

The failure of the reaction to appear until the 8th or 9th day makes one hesitate to accept the first of these three possibilities. Films show that streptococci are progressively decreasing in numbers in the lesion from the 2nd day onward. It is difficult to explain why a toxic product contained in the bacteria themselves should not exert its action earlier than the 8th day. It is, however, possible, that the disintegration of the streptococci or degradation of the bacterial substance to a stage where toxic material is liberated is a process requiring several days. A toxic product of the rabbits' tissues themselves cannot be excluded; if such exists it would seem to be a substance only called into existence at this particular time by a certain class of bacteria. The third possibility, that of a toxic product formed by the interaction of substances furnished by both invader and host, is more inviting. Analogies with serum sickness in man and the Arthus phenomenon in rabbits are at once obvious; and if we accept this interpretation, we

are almost compelled to classify the secondary reaction as an allergic phenomenon. We may suppose that the streptococci or some antigenic product of them persists at the site of inoculation until after the formation of antibodies by the rabbit, and that, just as in the Arthus phenomenon, the product of the antigen-antibody union, which then occurs, excites a local inflammation. A number of difficulties appear when one attempts to prove this hypothesis experimentally. They will be discussed in a later paper. Whatever may be the eventual explanation of the secondary reaction, certain features in it are of great interest. The failure of the phenomenon to appear after a second inoculation, at any rate if this is made within a limited period after a first, makes it very probable that the substance exciting the reaction is antigenic. During this refractory period the rabbit may possess an immunity to the irritating substance of such sort that it gives no secondary inflammatory response; or antibodies present in the tissues as a result of the first inoculation may unite immediately with the antigen, producing an inflammation which is masked by the more vigorous response of the animal to the injected streptococci; the primary and secondary reaction would thus occur simultaneously and be superimposed.

The most interesting feature of the phenomenon is its particular association with the streptococcus group. Since preliminary injection with any member of that group, in which we include the pneumococci, usually results in the prevention for a certain period of the occurrence of the secondary reaction following the inoculation of a strain ordinarily capable of exciting this reaction, it is probable that some preventing or antigenic substance is one common to the whole group. Whether this substance and that exciting the secondary reaction are one and the same is a matter for future study.

It may be that this secondary reaction is a curious phenomenon of academic interest only. On the other hand, there is much yet to be learned of the part which streptococci play in disease. The study of this skin response of rabbits may enable us to interpret the relationship between streptococci and their host from a new angle. Attempts have often been made to explain rheumatic fever, nephritis, and some of the sequelæ of scarlet fever in terms of allergy, particularly in relation to streptococci. This reaction, especially if it should prove to be a

form of allergy, may eventually have some bearing on complex problems such as these.

SUMMARY.

1. Rabbits inoculated intradermally with certain strains of green streptococci have well marked lesions which, after reaching a maximum size in 24 to 48 hours and then beginning to retrogress, show in over 50 per cent of the animals a secondary increase in size and other signs of inflammation about 8 or 9 days after inoculation.

2. This secondary reaction may follow the inoculation of a variety of strains of green-producing streptococci from various sources, of indifferent streptococci, and occasionally of pneumococci.

3. The inoculation of hemolytic streptococci, staphylococci, *Micrococcus catarrhalis*, or *Bacillus coli* has not been followed by this secondary reaction.

4. The secondary reaction is not due to increased activity of the injected bacteria since the lesions are usually sterile at the time it occurs and since the secondary reaction occurs after inoculation of killed organisms as well as after that of living ones.

5. A second inoculation of green streptococci into a rabbit within 9 weeks of a first injection is followed by a primary but not by a secondary reaction. This inhibition of the secondary reaction is not specific; for the phenomenon of secondary reaction can be completely inhibited by previous inoculations of the rabbit with any living streptococcus and usually with pneumococci. Killed organisms are less effectual.

6. Preliminary injection with staphylococci, *Micrococcus catarrhalis*, and *Bacillus coli* has not prevented the appearance of a secondary reaction in response to subsequent injections of green streptococci.

7. The agent responsible for the secondary reaction has not as yet been determined; its nature is discussed. The phenomenon is possibly a form of allergic reaction.

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EXPLANATION OF PLATE 2.

FIGS. 1 and 2. Lesions in two different animals 8 days after inoculation.

Each animal was injected at Site I with 0.1 cc. and at Site II with 0.02 cc. of culture on the same day.

The comparable lesions in the two animals were similar until the 7th day, but on the 8th day Animal 1 (Fig. 1) showed a marked secondary reaction in each lesion, whilst in Animal 2 (Fig. 2) there was no secondary reaction at any time.



FIG. 1.



FIG. 2.

THE SKIN RESPONSE OF RABBITS TO NON-HEMOLYTIC STREPTOCOCCI.

II. ATTEMPTS TO DETERMINE WHETHER THE SECONDARY REACTION IS OF THE NATURE OF AN ARTHUS PHENOMENON.

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INTRODUCTION.

In the preceding paper are described the lesions which follow the inoculation of certain non-hemolytic streptococci into the skin of the rabbit; such lesions begin to decrease in size after 24 to 48 hours but in over 50 per cent of animals show a recrudescence of activity about the 8th or 9th day. This phenomenon we have termed the secondary reaction. It usually fails to occur in animals which have been previously inoculated with any variety of streptococcus within a period of 9 weeks. Reasons have been given for attributing this secondary reaction either to a toxic product of the streptococci which for some reason takes a number of days to exert its action, or more probably to an antigen-antibody reaction, analogous perhaps to the Arthus phenomenon.

Arthus (1) noted that rabbits immunized against horse serum would show an acute local inflammatory reaction in response to a subcutaneous injection of horse serum which produced no such effect when injected into a normal animal. In a recent study of this phenomenon, Opie (2) concluded that the inflammatory reaction depended upon the formation of a toxic product when antigen and antibody were brought together in suitable proportions, because he found that the intensity of the Arthus phenomenon was roughly parallel to the intensity of precipitin formation. An inflammatory reaction resulted not only when antigen was injected into an animal the body fluids of which contained antibody, but also when antibody was injected locally into a rabbit which had very recently received an injection of antigen and which probably still contained that antigen in its body fluids; acute inflammation also followed the subcutaneous injection of an *in vitro* mixture of antigen and antibody.

Although it was shown by us that no bacteria could be grown from the skin lesions at the time of the secondary reaction, still it is easily comprehensible that antigen in the shape of dead microorganisms or degradation products thereof might still be present locally. With the knowledge that antibodies usually are first demonstrable about 8 days after a primary inoculation the hypothesis naturally suggested itself that the secondary reaction might be the result of the union of these antibodies and some locally persisting antigenic substance, which formed a new irritating compound. The fact that this reaction was demonstrable in rabbits gave additional support to an analogy with the Arthus phenomenon. In considering this hypothesis we fully realize that it is not always possible to apply our knowledge of other forms of allergy to that obtained with bacteria and bacterial products.

This possible analogy between the secondary reaction and the Arthus phenomenon seemed open to experimental study. If similar conditions held good in the two cases we should expect to find (1) that injections of streptococcal antigen into an animal having antibodies in its body fluids would provoke an inflammatory reaction not shown by a normal animal; (2) that injection of antibody into an animal the fluids or tissues of which contained streptococcal antigen would provoke an inflammatory reaction; (3) that mixtures of streptococci and antibody would give rise to more inflammation than was caused by either alone; (4) that some quantitative relation would be found between the occurrence of a secondary reaction in an animal and the antibody formation by that animal.

The technique used in the following experiments was the same as that described in the preceding paper.

EXPERIMENTAL.

I. Attempt to Alter the Reaction of a Rabbit's Skin to Green Streptococci by Giving Homologous Immune Serum Locally before Inoculation.

Two rabbits received intradermally $\frac{1}{2}$ cc. of immune rabbit serum produced by weekly intradermal immunization; six injections in all had been given in the process of immunization and the resulting agglutinin titre of the serum against Strain V92 was 1:2560. On the following day live green streptococci, Strain V92, were inoculated into the site of the serum injection. The lesions resulting were of

approximately the same size (50 and 55 mm.) as in two control animals (52 and 53 mm.). Moreover, the serum had apparently no inhibiting effect on the development of a secondary reaction, as one of the two animals had a typical secondary reaction at the usual time, that is on the 9th day.

II. Attempt to Precipitate a Secondary Reaction by Giving Immune Serum Intravenously a Few Days before the Reaction Was Due.

Two rabbits were given an intravenous injection of 5 cc. of antistreptococcus immune serum similar to that used in Experiment I, 5 days after they had received an intradermal injection of green streptococci (V92). No immediate reaction occurred at the site of the skin lesions. One of the two showed a secondary reaction at the usual time (10th day); the other showed no secondary reaction. Since it was possible that 5 cc. might be an insufficient quantity of serum, two other rabbits were each given 45 cc. of immune serum intravenously 4 days after intradermal inoculation; this serum was also prepared by intradermal immunization of rabbits, but had an agglutinin titre of only 1:640 against *Streptococcus* V92. Results similar to those observed in the first group were obtained. Neither animal showed an accelerated secondary reaction; one showed no secondary reaction; the other gave a somewhat late reaction on the 13th day.

III. Attempt to Precipitate a Secondary Reaction by Giving Immune Serum Locally a Few Days before the Reaction Was Due.

By giving immune serum at the site of the lesions, it was hoped to obtain a greater local concentration of antibodies than was possible after intravenous passive immunization. Three animals were, therefore, injected intradermally in two places with green streptococci; 5 days later, one rabbit, and the following day the other two, were each given 0.2 cc. of homologous immune serum at the site of one lesion and 0.2 cc. of normal rabbit serum at the site of the other. Aside from the slight local reaction usually seen in any rabbit when serum is injected intradermally, no rabbit showed any immediate response suggesting a premature secondary reaction. Two of the rabbits showed no secondary reaction; the third showed typical reactions in both lesions on the 9th and 10th days respectively.

IV. Effect of Injection of Mixtures of Streptococci and Immune Serum into Normal Rabbits' Skins.

The sediment of 5 cc. blood broth culture of green streptococci, Strain V92, was mixed *in vitro* with immune sera prepared by intravenous or intradermal inoculation of rabbits with the homologous organism. The mixture was inoculated into four rabbits. For controls, mixtures of bacteria with saline, bacteria with normal rabbit serum, and bacteria with heterologous green streptococcus immune

serum were used. The maximum size of the lesions at the end of 24 hours is shown in Table I.

It is evident from Table I that the lesions resulting from mixtures of bacteria and immune serum were no larger than those following inoculation of organisms alone, or of mixtures of organisms and normal

TABLE I.

Effect on the Size of the Lesions Produced in Rabbits' Skins of Mixing Various Sera with the Streptococci Injected.

Rabbit No.	Serum used.	Size of lesion (sum of diameters)..
		<i>mm.</i>
1	None.	39
	Normal rabbit.	38
	Homologous streptococcus.*	27
	" " †	32
2	None.	47
	Normal rabbit.	36
	Homologous streptococcus.*	30
	" " †	26
3	None.	39
	Normal rabbit.	35
	Homologous streptococcus.*	34
	Heterologous " *	29
4	None.	43
	Normal rabbit.	34
	Heterologous streptococcus.*	21
	" " †	21

* Immune serum produced by intravenous inoculation.

† " " " " intradermal "

serum. They were, in fact, always slightly smaller than the controls. Measurements of these lesions at the end of 48 and 72 hours, and of lesions resulting from inoculation of the sediment from 0.5 and 0.05 cc. of culture with the same amount of serum confirmed these results. If this is evidence of any protective power in the serum it was apparently not specific. This point was, however, not studied in detail.

V. Attempts to Correlate the Appearance of the Secondary Reaction with the Production of Immune Bodies.

We next endeavored to determine whether immune bodies could be demonstrated in a rabbit's serum after one intradermal injection of the sediment from 5 cc. blood broth culture streptococci, and whether the degree of antibody formation was related in any way to the second-

TABLE II.

Relationship between Agglutinin Production and Occurrence of Secondary Reaction.

Rabbit No.	Green streptococcus used.	Secondary reaction.	Day on which secondary reaction was maximal.	Agglutinins present.					
				Before inoculation.	Days after inoculation.				
					6	8	9	10	11
1	Strain T4	+	10	0	0	.	++	.	.
2	" V110A	+	11	.	.	.	++	.	.
3	" V110A	+	10	.	.	.	0	.	.
4	" V92	+	8	0	.	.	.	0	.
5	" V92	+	6	0	.	.	.	0	.
6	" V92	+	9	0	.	0	0	.	0
7	" V92	±	10	0	.	0	++	.	++
8	" V92	±	9	0	.	.	.	0	.
9	" V92	±	7	0	.	.	.	0	.
10	" V110A	±	10	.	.	.	0	.	.
11	" T4	0	—	0	+	.	++	.	.
12	" V110A	0	—	0	0	.	0	.	.
13	" T4	0	—	0	0	.	0	.	.
14	" V92	0	—	0	.	.	.	0	.
15	" V110A	0	—	0	0	.	0	.	.

In the agglutinin column ++ means agglutination up to 1:80 to 1:160.

ary reaction. Agglutinins were chiefly studied; for obtaining stable suspensions of streptococci we used the technique described by Dochez, Avery, and Lancefield (3).

The results summarized in Table II indicate that there was no absolute correlation between the ability of a rabbit to give a secondary reaction and its power to form agglutinins in demonstrable concentration. Thus, in only two out of six rabbits showing a definite secondary reaction was it possible to demonstrate agglutinins on the

TABLE III.

Relationship between Agglutinin Production and Occurrence of Secondary Reaction.

Rabbit No.	Time of bleeding with reference to inoculation.										Relative strength of agglutinin production.
	Days before.	Days after.									
		1	5	6	7	8	9	10	11	14	
1	1	3	.	4	.	9	.	12	.	17	12
	.	+++	+++	++++	++++	+++	+++	+	.	.	
2	0	.	1	.	8	.	2	.	7	.	18
	.	+	++++	+++	+++	++	+	.	.	.	
3	4	.	12	.	13	.	19	.	14	.	2
	.	.	.	++++	++++	++++	++++	(c)	.	.	
4	0	.	9	.	4	.	9	.	11	.	15
	.	.	.	+	++	++++	++++	±	.	.	
5	0	.	.	10	.	13	.	11	.	17	8
	++	++++	++++	++++	.	.	
6	0	.	.	13	.	13	.	18	.	6	3
	.	.	.	+	+++	+	±	.	.	.	
7	0	.	16	.	15	.	11	.	11	.	5
	.	.	+	++	±	±	
8	2	.	.	14	.	19	.	19	.	15	1
	++	++	+	+	±	.	
9	4	.	.	9	.	15	.	18	.	11	4
	.	.	.	++	+	
10	0	.	3	.	7	.	10	.	9	.	14
	.	.	.	+	++	

Agglutinin strength indicated by figures (see text). (?) means serum lost.

Intensity of secondary reaction indicated as follows:

0 indicates no secondary reaction.

± " an increase of less than 3 mm.

+ " " " 4-6 mm.

++ " " " 7-9 "

+++ " " " 10-12 "

++++ " " " 13 mm. or more.

(e) means lesion excised.

TABLE III—*Concluded.*

Rabbit No.	Time of bleeding with reference to inoculation.										Relative strength of agglutinin production.
	Days before.	Days after.									
		1	5	6	7	8	9	10	11	14	
11	0	.	.	10	.	8	.	10	.	6	13
	+	++	.	.	.	
12	0	3	.	7	.	(?)	.	13	.	6	11
	++	
13	0	.	1	.	2	.	4	.	6	.	20
	+	+	+	+	.	.	
14	0	.	6	.	9	.	15	.	12	.	6
	+	+	
15	0	.	9	.	11	.	13	.	17	.	10
	+	+	.	.	.	
16	0	.	10	.	13	.	12	.	12	.	9
	+	±	.	.	.	
17	0	.	.	3	.	(?)	.	1	.	2	21
	±	+	.	.	.	
18	0	3	.	3	.	1	.	9	.	5	17
	.	.	.	±	±	
19	1	.	.	13	.	14	.	(?)	.	11	7
	±	
20	0	.	7	.	9	.	8	.	13	.	16
	.	.	0	0	0	0	0	0	0	.	
21	0	.	1	.	1	.	6	.	6	.	19
	.	.	.	0	0	0	0	0	.	.	

9th or 10th day; in one out of four rabbits giving a doubtful secondary reaction equally as strong agglutinins were present on the 9th and 11th days, while the other three developed no agglutinins at the time of the secondary reaction. Among five rabbits not showing secondary reac-

tions one gave a good agglutinin titre, the other four gave none. While a lack of absolute correlation between these two reactions is indicated by this table, there seems to be some rough parallelism between the proportion of rabbits giving both reactions positive and both negative. The criticism can, however, be made that the sera of these animals was not tested frequently enough to demonstrate immune body formation.

A more extensive single experiment was, therefore, performed to test this point.

Twenty-one rabbits were each inoculated in two places with the sediment of 5 cc. of 18 hour culture of Strain V92 and the course of the skin reaction charted as usual. All of the animals were bled before inoculation; half of them were subsequently bled on the 6th, 8th, 10th, and 14th days, the other half on the 7th, 9th, 11th, and 15th days respectively; when secondary reactions occurred before this period the rabbits were also bled at this earlier time. All of the sera were stored in the cold, and tested simultaneously for agglutinins against the same suspension of Strain V92. Each serum was diluted 1:20, 1:40, 1:80, 1:160, and incubated 2 hours at 56°. Immediate readings were made and recorded. Tests repeated on 2 different days confirmed one another. For purposes of comparison the number of + marks obtained with each serum were added, the sum multiplied by the factor 2 to eliminate fractions, and the final product entered as the intensity of agglutinin formation for each animal on a given day. While this method is not absolute it does give a good comparative index of agglutinin content of each serum. The intensity of the secondary reaction is charted by a series of pluses. We thus have a comparison of the agglutinin curve in each rabbit with the intensity of the secondary reaction over its entire period.

In Table III the results obtained with various animals are arranged from above downward according to the time of appearance, intensity, and duration of the secondary reaction in each rabbit. In the right hand column is shown the comparative intensity of agglutinin formation of each rabbit during the period of observation. Although a very rough correlation between the intensity of the two reactions may possibly be seen, a detailed analysis indicates that three of the five rabbits having the most marked secondary reactions were below the median in agglutinin formation; while four of the seven rabbits showing a + or ± secondary reaction were above the median. It is unusual for such a small proportion of rabbits to have no second-

ary reaction; hence the experiment may be criticized from the standpoint of negative control; but the results in general indicate a lack of correlation between the formation of humoral antibodies and the intensity of the secondary reaction.¹ This was also confirmed by testing these sera for precipitins against nucleoprotein and soluble specific substance prepared from Strain V92, when a complete disagreement between precipitin formation and secondary reaction was found.

DISCUSSION.

In five different types of experiments we have attempted to test the hypothesis that the secondary reaction might be explained on the lines of a local anaphylaxis comparable with the Arthus phenomenon. In none did we obtain any support of this hypothesis. No accelerated reaction was brought about in infected animals by introducing immune serum intravenously or locally; antigen-antibody mixtures produced no unusual reactions; no relation could be found between the secondary reaction and agglutinin or precipitin production.

In view of what has already been demonstrated by Zinsser (4) and his coworkers concerning the tuberculin reaction and its relation to antibody formation on the part of a tuberculin-sensitive animal, it was perhaps too much to expect that the Arthus phenomenon would find a close analogy in the cutaneous phenomena associated with an infection. In one case we are dealing with a coagulable protein, in the other with bacteria or bacterial products; hence we are not justified in concluding from our negative results that the secondary reaction is not allergic in nature. It is, indeed, well recognized that two types of hypersensitive reactions may be demonstrated in the skins of animals: in one the response is rapid and largely exudative in nature, best exemplified in the urticarial wheal; in the other the response is slower in appearance and characterized by more proliferation of the fixed tissue elements. We are unable to state from our experiments that the proliferative response seen at the time of the secondary reaction is not

¹ It is probable that the difference in the number of negatively reacting animals summarized in Tables II and III is due to the fact that the first group received a single inoculation while the second group received two. We have recently demonstrated that results are much more uniform when two inoculations of 5 cc. each are given.

due to the union of some product of the bacterial cell and an antibody present in the tissue cells, which antibody we are unable to recognize and work with in the same manner as we can with the usual humoral antibodies. In any event it would seem that the secondary reaction is analogous rather to the tuberculin reaction than to the Arthus phenomenon, and experiments are now under way with this last viewpoint in mind.

SUMMARY.

An attempt has been made to interpret on the lines of the Arthus phenomenon the secondary reaction which follows the intradermal inoculation of certain non-hemolytic streptococci into rabbits; but evidence in support of this interpretation of the reaction has not been obtained. The facts make it seem probable that if this secondary reaction is due to a hypersensitive state it must be one more closely allied to the tuberculin reaction than to the Arthus phenomenon.

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THE FILLING AND EMPTYING OF THE GALL BLADDER.

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PLATE 3.

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The discovery of a means of visualizing the gall bladder by the Roentgen ray after intravenous injection of certain halogen derivatives of phenolphthalein by Graham and Cole (1) has given a new means of studying the physiology of the biliary tract. This article belongs to a series of experimental and clinical reports (2) relating to that subject and will be particularly concerned with the filling and the emptying of the gall bladder. The experimental work has been done by both cholecystography and other laboratory methods.

A resistance to the outflow of bile from the common duct was found by cholecystography to be necessary for the collection of bile by the gall bladder (3). This resistance to the outflow of bile from the common duct into the duodenum was later found to be largely due to tonus and movements of the duodenum (4). As a result of this regulatory mechanism at the distal end of the common duct there are considerable changes of pressure in the gall bladder and the rest of the biliary tract which not only aid in the filling of the gall bladder but are a factor in its emptying. In this article we shall refer to this mechanism for the regulation of the flow of bile at the distal end of the common duct as the common duct sphincter.

The pressure changes that are reflected over the rest of the biliary tract by the opening and closing of the common duct sphincter have been demonstrated in the etherized dog in the following way.

A cannula which was inserted into the middle hepatic duct was connected with a supply of physiological saline solution for making pressure in the system. The continuous flow of bile in the hepatic ducts from the liver of man and dogs is under secretory pressure. A tambour connected by a T-tube with the cannula

was used to record changes of pressure in the hepatic duct on a smoked drum. Records of changes of pressure were also made by tambours connected with the gall bladder and with the common duct. Lateral hepatic ducts were ligated. When the common duct is closed, as if by the action of a sphincter, the pressure in the duct quickly rises. After a short delay, the pressure in the gall bladder rises. The pressure in the hepatic duct rises simultaneously with that in the gall bladder. If the common duct is opened, there is an immediate fall of pressure in it which is followed shortly and consecutively by a fall of pressure in the gall bladder and hepatic duct. We have recorded a drop of pressure in the gall bladder occurring at the same time with the relaxation phase of a peristaltic movement in the duodenum. This experiment was performed by ligating the hepatic ducts and simultaneously recording the pressure in the gall bladder and peristalsis in the duodenum.

These changes of pressure in the biliary tract are exactly similar to the changes of pressure that have been demonstrated by one of us (5) in a model designed to show the changes of pressure which normally take place in the biliary tract.

The control of the contents of the gall bladder by the common duct sphincter is further demonstrated by cholecystography.

The stomach or duodenum of man or animal must be free from food in order to secure a cholecystogram after administration of sodium tetraiodophenolphthalein. If digestion is in progress, bile is permitted to enter the duodenum too freely by the common duct sphincter; and the iodine in the bile will not enter the gall bladder to produce a shadow. Likewise, a cholecystogram is not obtained if the common duct sphincter of the dog is kept open by the insertion of a cannula into the distal end of the common duct. The bile laden with the halogen passes directly through the hepatic and common duct to the duodenum. A cholecystogram may be obtained, however, after the ligation of the hepatic duct of a middle lobe of the liver of a dog. The bile from the other lobes, containing the iodine, enters the common duct from the lateral hepatic ducts, which, in the dog, enter the common duct distal to the cystic duct, and is dammed back into the gall bladder by the closure of the common duct sphincter.

A factor in the prevention of the overdistension of the gall bladder by continuous accessions of bile is the S-shaped portion of the neck of the ampulla and cystic duct. Attention has been called to this fact by Jacobson and Gydesen (6). When there is considerable pressure in the fundus of the gall bladder, the S-shaped portion kinks upon itself and tends to prevent the exit or the entrance of more bile. The valves of Heister in the cystic duct do not normally seem to offer much resist-

ance to the entrance or exit of bile. They are doubtless more efficient as a control when the kink of the cystic duct is present. The ampulla and cystic duct are of great clinical importance.

4 or 5 hours after the intravenous injection of sodium tetraiodophenolphthalein, sufficient iodine will enter the normal gall bladder of a fasting man or dog to cast a shadow on a film by the Roentgen ray. The shadow in 4 or 5 hours becomes smaller and is densest between 16 and 24 hours after injection. The shadow tends to come to a minimum size and gradually fades away by the end of 30 to 40 hours.

We found when investigating the appearance and disappearance of the shadow of the gall bladder that the halogen in the bile is concentrated two to four times by the gall bladder (3). This fact confirmed the work of Rous and McMaster (7) who demonstrated the concentration of bile pigment by the absorptive power of the gall bladder. It was further found that the greater part of the contents of the gall bladder leaves its lumen by way of the cystic duct (3). This latter fact has also been demonstrated by Whitaker (8). However, this conclusion is in contradistinction to the opinions of Sweet (9), Halpert (10), and Demel and Brummelkamp (11), who believe that whatever enters the gall bladder through the cystic duct does not normally pass out of it by way of the cystic duct.

Factors of dilution seem of great importance in the appearance and disappearance of the shadow of the gall bladder. If all of the hepatic ducts are ligated, leaving the common duct intact, after a dense shadow of the gall bladder has been secured from tetraiodophenolphthalein, the shadow will be found to persist for many days even while food is being taken. It is inferred from this experiment that dilution of the bile in the gall bladder by bile entering from the hepatic ducts is an important factor in the filling and emptying of the gall bladder. Ordinarily, during a cholecystographic examination, the course of events is somewhat as follows:

The tetraiodophenolphthalein is excreted in the bile shortly after injection. The sphincter of the common duct diverts a considerable portion of the bile into the gall bladder. The amount of bile secreted is probably not large, comparatively, since the animal is fasting. The addition of iodine to the bile in the gall bladder proceeds. The gall bladder becomes distended. At this time we may obtain our first shadow of the gall bladder. It is a large and faintly visible one.

The amount of iodine in the gall bladder increases by new additions of bile as the concentrating activity of the gall bladder proceeds simultaneously. The iodine content becomes great enough to cast the densest shadow in 16 to 20 hours after injection of the dye. During this same period, the iodine has probably been entirely excreted by the liver. Since iodine is no longer present in the bile coming from the liver, the shadow gradually disappears as dilution of the iodine in the gall bladder progresses. In the meantime, food has been given to the patient and the disappearance of the shadow is hastened by an increased secretion of bile and a relaxed common duct sphincter. The backing up of dilute bile from the liver into the gall bladder mechanically aids in the expulsion of the concentrated bile from the gall bladder.

Changes of density of the cycle of the shadows of a gall bladder that normally occur after administration of tetraiodophenolphthalein may be duplicated outside of the body by the use of a rubber bag containing sodium iodide. A small thin rubber bag approximately the size of a gall bladder is attached to the arm of a T-tube. The changes in the shadows of a gall bladder can be duplicated by varying the size of the bag and by the dilution of its content of sodium iodide.

It was suggested by the preceding experiments of ligating the hepatic ducts and by other facts that the gall bladder is never entirely empty. Such was found to be true. The shadow of the gall bladder of a dog will always be present if he is given a daily dose of tetraiodophenolphthalein and films made at intervals. It persists even though he is fed regularly and allowed normal freedom of action. The shadows vary in size but tend to reach a minimum size.

The finding that the gall bladder is never empty is consistent with pressure changes in the biliary tract. The pressure in the gall bladder tends to come to an equilibrium with the rest of the system though it is not necessarily the same. Bile will pass out from a distended gall bladder if the common duct sphincter is open. Also, if the sphincter is open, bile coming from the hepatic ducts may assist in the emptying of the gall bladder both by dilution and by tending to produce a negative pressure in the gall bladder in its passage down the ducts through the open sphincter into the duodenum, in a manner similar to the action of the filter pump. When its content is at a minimum pressure the gall bladder will not discharge bile even though it is not entirely empty.

The fact that the gall bladder is never entirely empty coincides with

the well known clinical fact that the gall bladder is never found empty on abdominal exploration. The physiological importance of this fact may be explained by the suggestion of Boyden (12) that the capillary network of the rugæ of the gall bladder of the cat is greater in collapse than when pressed out by distension. He believes that the most rapid absorption takes place when the gall bladder is partially collapsed. It is during partial collapse of the gall bladder that the density of the shadow of a cholecystogram is maximum. The gall bladder is in this state of partial collapse for the longest period of time during its visibility by Roentgen ray.

We have noted that the shadow of the normal gall bladder varies in size, denoting that other factors than dilution or interchange of bile may play a part in the slow emptying of the gall bladder. The importance of changes of intraabdominal pressures, especially due to respiration, has been lately stressed by Winkelstein (13). The effect of respiration on the intravesical pressure may be demonstrated by placing a manometer in the gall bladder and closing the abdominal wall about it as was done in the anesthetized dog. There may be a variation as high as 60 mm. of bile during respiration, vomiting, and other changes of intraabdominal pressure. The changes of pressure in the ducts with respiration are not so large as those in the gall bladder. Bile in some instances may be seen to be expelled from the duodenal papilla with forced respiration. Furthermore, the shadow of a gall bladder in a dog may be decreased in size by deep palpation of the abdominal wall over the region of the gall bladder.

We believe with Boyden (12) that, as regards emptying, the gall bladder is largely a passive organ, except for the elasticity of its walls. It is influenced by pressure changes which we have described. The anatomy of the walls of the gall bladder are ideal for such an elastic or contractile mechanism. Its tonus varies in response to pressure changes. The smooth muscle fibers and connective tissue aid in preventing overdistension and prevent complete collapse of the walls. The distensible walls equalize extremes of pressure due to the variation of the rate of secretion of bile, to the variable state of contraction of the common duct sphincter, and to changes of intraabdominal pressure. The elasticity of the walls is also a mechanism for the expulsion of bile through the cystic duct. The maximal pressure which the wall

of the gall bladder can exert is difficult to determine *in situ*. It is probably great enough to expel bile from a distended gall bladder when the common duct sphincter is relaxed. Though the gall bladder has an elastic or contractile mechanism which exerts pressure on its contents until it has reached a minimum pressure, we have not been able to demonstrate an actively contracting movement due to its intrinsic musculature. Direct fluoroscopic examinations of visualized gall bladders have uniformly failed to show any evidence of a peristaltic wave; and more important still, direct electrical stimulation of the wall of the gall bladder of an anesthetized dog has never resulted in a contraction wave, although a similar stimulation of the intestine induced violent peristaltic contractions. This agrees with a previous observation made by Boyden (12) and Whitaker. Rhythmic changes of pressure of the content of the gall bladder have been recorded by us by various means in the anesthetized dog. However, no curves have been obtained from the gall bladder that have not been duplicated by a rubber bag of like size placed in the upper part of the abdominal cavity of the etherized dog. The rhythmic changes which were recorded, corresponded to changes of intraabdominal pressure largely due to respiration.

The same experiment as the preceding one was conducted on dogs after operation under ether anesthesia.

A rubber tube was connected to a small rubber bag which was placed in the fundus of the gall bladder. A similar bag was placed alongside the viscera of the upper abdomen. The rubber tubes were brought out of the abdominal wall through different incisions. The dogs were allowed to recover from their operations. The bags were distended with air and simultaneous readings made of the changes of pressure after feeding, alteration of position of the dog, etc. The records from each bag corresponded essentially. This experiment confirms the finding in the preceding one that the so called rhythmic contractions were independent of peristaltic or other active muscular contractions of the wall of the gall bladder.

There has not been an indication of an actively effective contraction of the gall bladder, such as a peristaltic movement of the intestine or evacuation of the urinary bladder, in the thousands of cholecystograms that have been made by this department in man and animals. All of the changes in size and shape of the shadows are referable to

an expansile organ. It is not necessary in any instance to explain the emptying of the gall bladder on the basis of an active muscular contraction of its wall.

Further proof of this rather passive reaction of the gall bladder is offered by the results obtained from an artificial gall bladder. A thin rubber bag the size of a gall bladder may be tied into a cystic duct of a dog by means of a short glass tube after cholecystectomy. If the animal is given a dose of sodium tetraiodophenolphthalein after the surgical operation, there will be a faint shadow of the artificial gall bladder visible in 4 hours. 16 hours later the shadow is larger and denser. The shadow may persist 6 or 8 days. The cycle of shadows produced by the artificial, rubber gall bladder is quite like that obtained from the normal gall bladder of the human, except that the shadows may not be so dense and that they usually persist over a longer period of time (Fig. 1, *a* and 1, *b*). The presence of the shadow seems to indicate that a forceful contraction due to a muscular action of the gall bladder wall is not necessary for the emptying or filling of the gall bladder. While the elastic or contractile mechanism present in the gall bladder is not necessary for its filling or emptying, it is probably the lack of this factor in the artificial gall bladder which delays its emptying. In this experiment, the increase in density of the shadow obviously cannot be due to concentration of the bile in the rubber bag but more probably must be due to a greater amount of iodine which has entered the bag through the cystic duct in its process of filling. In ordinary cholecystography the increase in density of the shadow which is observed is probably due in part to the factor of concentration by absorption of water. The shadow in the artificial gall bladder appears quite as promptly as in the normal gall bladder. The disappearance of the shadow must take place by washing out of the bile in the bag by fresh liver bile and by intraabdominal pressure. The pressure in the biliary tract is not sufficient to distend the rubber bag. Also, since the artificial gall bladder is not in apposition to the liver, changes in volume of that organ are not necessary for the filling or emptying of the gall bladder, although doubtlessly they are normally a factor. Changes of blood pressure in the normal gall bladder do not seem to be essential for its activity. This experiment, furthermore, casts considerable doubt on the contrary innerva-

tion of the gall bladder described by Meltzer (14). He has concluded that there is a coordinated action of relaxation of the sphincter of Oddi and contraction of the gall bladder. Lyon (15) has based the nonsurgical drainage of the gall tract upon this hypothesis.

CONCLUSIONS.

As a result of the control of the flow of bile into the duodenum largely by tonus and movements of the duodenum, bile intermittently enters the gall bladder where it is concentrated and undergoes other changes. The gall bladder empties itself of its content through the cystic duct (1) by the washing out of its contents by bile from the liver, (2) by the elasticity or contractile mechanism of its walls, and (3) by variations of intraabdominal pressure due to respiratory movements, contiguous organs, etc. A fourth manner of emptying of the gall bladder is by absorption of a portion of its content through its walls. The gall bladder is never entirely empty but tends to come to a state of partial collapse, when its contents are under minimum pressure. We have been unable to demonstrate rhythmic contractions of the gall bladder due to its musculature. If they are present they may aid but they are not essential for its emptying or filling. Experimentally, in the dog, a rubber bag which was substituted for the gall bladder functioned in a manner very similar to that of the normal gall bladder as shown by cholecystographic studies. The concentrating function, however, was absent.

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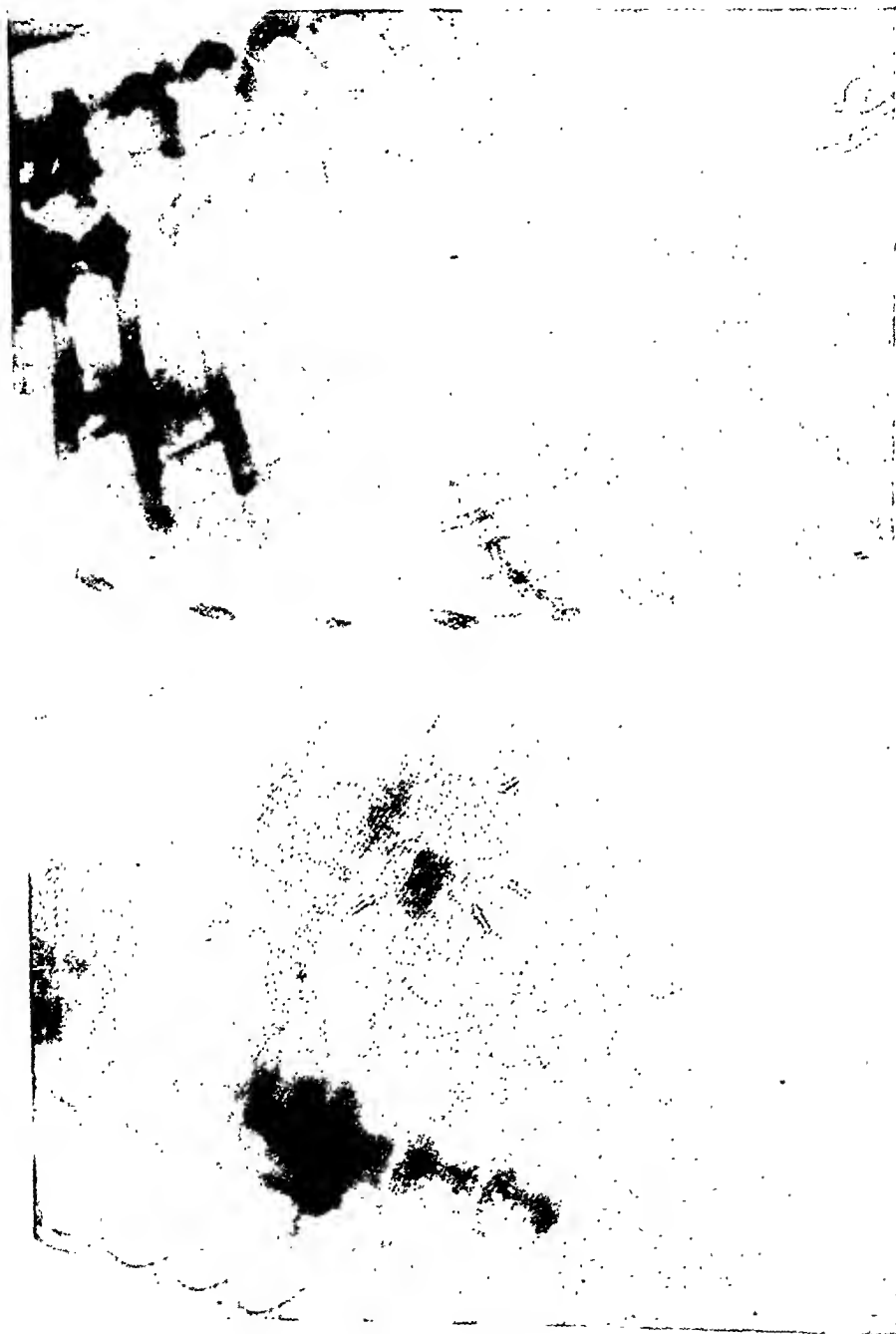
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EXPLANATION OF PLATE 3.

FIG. 1, a. Arrows point to the shadow produced by an artificial, rubber gall bladder, 24 hours after intravenous injection of sodium tetraiodophenolphthalein. The small dense shadow within the shadow of the gall bladder is the glass tube which connected the bag with the cystic duct. The same tube shows in Fig. 1, b.

FIG. 1, b. Roentgenogram of the same dog 6 days later. The shadow of the artificial gall bladder has disappeared.



(Copher, Kodama, and Graham: Gall bladder.)

THE RELATION OF THE RETICULO-ENDOTHELIAL SYSTEM TO THE BLOOD PLATELET COUNT.

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In studying the diminution in the number of blood platelets which is one of the most prominent factors in the pathogenesis of thrombocytopenic purpura, the author could not find positive evidence in the literature, of the cause of this thrombopenia. Many conflicting opinions were encountered, and these could be narrowed down to two basic ones. Frank and Glantzmann assume that the megacaryocytic apparatus is injured or inhibited from producing the normal number of platelets. Kaznelson and many others support the opposite view; namely, that there is an excessive thrombolytic function in the spleen and perhaps in other parts of the body, which causes a marked diminution in the number of platelets, not by interfering with their formation but by increasing their destruction. It has already been established that splenectomy in the experimental animal is followed by a rise in the number of platelets in the blood (Bedson). The spleen is therefore supposed to be the site of destruction of quantities of platelets, and its removal inhibits much of this destruction. This rise, Bedson has shown, is temporary, the return to normal being completed after about 1 month. The explanation advanced for the gradual return to normal is that the hyperactivity of other physiologically related tissue compensates for the loss occasioned by splenectomy. The hyperactivity is manifested by an increased platelet destruction and the hitherto increased number of platelets (as a result of splenectomy) is diminished to the normal level.

It has also been noted clinically that splenectomy in thrombocytopenic purpura is rapidly followed by a rise in the platelet count. This relatively high count soon becomes diminished however, the fall in

some cases almost reaching the low level before operation. Is it because of a similar compensatory activity of physiologically related tissue?

It becomes evident that investigation regarding the source of supply and the mechanism of destruction of the platelets is necessary to clear up the haze which surrounds these problems.

The investigations of Ribbert, Aschoff, Kiyono, Goldmann, and others have established the existence of a group of cells widely distributed throughout the interstitial and perivascular tissues of the body, possessing common morphological and physiological properties, which they have grouped as a tissue under the heading of the reticulo-endothelial system. To the activity of this tissue, of which the spleen contains a large amount, has been attributed the destruction of platelets, and the investigation along one line of the basis for such assertion furnishes the subject matter of this paper.

One of the most prominent characteristics of these cells is their propensity for absorbing and storing in granular form vital stains introduced into the blood in solution. They phagocytose these vital stains voraciously, and becoming gorged, they are rendered otherwise relatively inactive. This method of blocking the system has been widely utilized to determine indirectly the activities of these cells.

In this study of the possible relationship between the reticulo-endothelial system and platelet destruction, the blocking system was used, platelet counts being made before and after the inactivation and rather striking results were obtained.

Method.

Guinea pigs were used in all the experiments. Before proceeding with the main problem, the method of platelet counting was studied. It was found that widely different platelet counts could be obtained by the same observer, using the same method, the same pig, during the same hour, the difference depending upon the manner in which the drop of blood was obtained.

The exertion of pressure on the surrounding tissue after needle puncture, to secure a large enough drop of blood for the procedure, is uniformly productive of a count at considerable variance with that obtained when the blood flows freely from the injured surface. Sticking the ear lobe in the attempt to strike the analogue of the marginal vein of the rabbit almost always fails to give a sufficiently large sized drop, as does needle sticking elsewhere in the body. It

was found that the best way to secure a good drop was by incising the foot with a safety razor blade, after thorough cleansing.

Various methods of counting were experimented with. They may be classified as direct and indirect. The first is self-explanatory. The second is accomplished by the determination in a blood smear of the relation between the amount of platelets and the number of red cells, and then the number of red cells per c.mm. is counted, from which the actual number of platelets is estimated. Probably the best method of this group is that of Bedson. The direct method seemed preferable because the possibility of error is apparently less. Various staining solutions were used (Wright and Kinnicutt, Leak and Guy, Reese and Ecker, etc.), but it was finally decided that the source of error would be minimized by counting the platelets unstained, because in the process of staining, despite all caution and effort to prevent it, very frequently small particles of stain would

TABLE I.

Guinea Pig No.	Platelet count.					Weight.			
	Feb. 4	Feb. 5	Feb. 6	Feb. 7	Average for 4 days.	Feb. 4	Feb. 5	Feb. 6	Feb. 7
						gm.	gm.	gm.	gm.
1	286,000	364,000	292,000	310,000	313,000	458	404	413	402
2	336,000	338,000	288,000	314,000	319,000	446	396	407	412
3	251,000	234,000	174,000	262,000	230,500	467	430	450	511
4	324,000	216,000	386,000	364,000	322,500	547	506	514	531
5	306,000	324,000	362,000	260,000	313,000	439	425	445	451
6	336,000	316,000	330,000	332,000	328,500	428	413	410	400
Total average.....					304,416				

be precipitated and these simulated platelets, thus interfering with accurate counting. The method adopted was to use a red cell counting pipette, draw up to the 1 mark, add 3 per cent sodium citrate to the 101 mark, and then after thoroughly mixing to place a suitably sized drop in a counting chamber, allow to settle for 15 minutes, count the platelets in 400 squares, and multiply the result by 1000. Because of the personal equation variation all counts were made by the same observer.

EXPERIMENTAL.

Six guinea pigs of approximately the same size were obtained from one source. They were segregated and watched for 4 days for signs of ill health. None being manifest, daily platelet counts were made on each one, over a period of 4 days. The counts ranged from a low level of 174,000 to a high level of 386,000. The average count for each pig was established as was also the total average. The latter was 304,416. (See Table I.)

Then 5 cc. of a $\frac{1}{2}$ per cent solution of trypan blue in freshly distilled water, sterilized by boiling, was injected subcutaneously into each animal. The trypan blue used in these experiments was obtained from the A. H. Thomas Co., of Philadelphia, some products of other derivation having been found highly toxic and therefore undesirable. Daily counts by the same technique were continued and the injections repeated 4 days later. On that day Pig 67 died. 9 days after the first trypan blue injection the count level had so changed that now the low level was 708,000 while the high reached 1,500,000. The total average for a period of 4 days, begun 8 days after the first trypan blue injection was 984,000, showing an increase over the total average, before injection, of 685,000. (See Table II.)

Very frequently during the experiment, check was made upon the platelet count and the degree of error was found to be very slight, being less than 5 per

TABLE III.

February 23. Injection of 5 cc. of $\frac{1}{2}$ per cent solution of trypan blue in each pig.

Guinea Pig No.	Platelet count.						Weight.					
	Feb. 23	Feb. 24	Feb. 25	Feb. 26	Feb. 27	Mar. 1	Feb. 23	Feb. 24	Feb. 25	Feb. 26	Feb. 27	Mar. 1
							gm.	gm.	gm.	gm.	gm.	gm.
1	708,000	844,000	694,000	728,000	676,000	576,000	483	489	475	470	468	482
2	694,000	468,000	512,000	460,000	402,000	364,000	380	303*	342*	320*	331*	343
3	776,000	620,000	808,000	796,000	622,000	760,000	474	480	465	470	465	484
4	640,000	604,000	802,000	940,000	776,000	762,000	507	540	517	518	522	540
6	984,000	864,000	636,000	662,000	548,000	608,000	487	478	482	497	511	536

* Diarrhea.

cent. Bedson and Zilva rate the possibility of error in counting platelets by their method at about 10 per cent. The estimated degree of possible error in this work bears out the previously expressed view that the direct method of counting is by far more desirable. However, in the light of the marked differences in counts obtained before and after the blockade, it is readily seen that even a much more generous allowance for error would not vitiate the results.

Two control animals, of similar stock, cared for under similar conditions, received injection of 5 cc. of distilled water at the same time that the test animals received the trypan blue injections. Those before injection showed a total average of 288,000 whilst the total average for the 4 days beginning 8 days after the first injection of distilled water was 278,000. Thus the rise in platelets in the other series is undoubtedly related to the injection of trypan blue.

The platelet counts were continued as shown in Table III. Apparently after the initial high excursion, there is a slight recoil and then a slow diminution in their numbers. (See Table III.)

Thus, after injection of trypan blue the platelet count shows a marked rise, the total average increase equalling approximately 300 per cent. This is followed by a sharp recoil and then a more gradual decline, despite another injection of trypan blue to continue the saturation. A graphic representation of the variation is given in Chart 1.

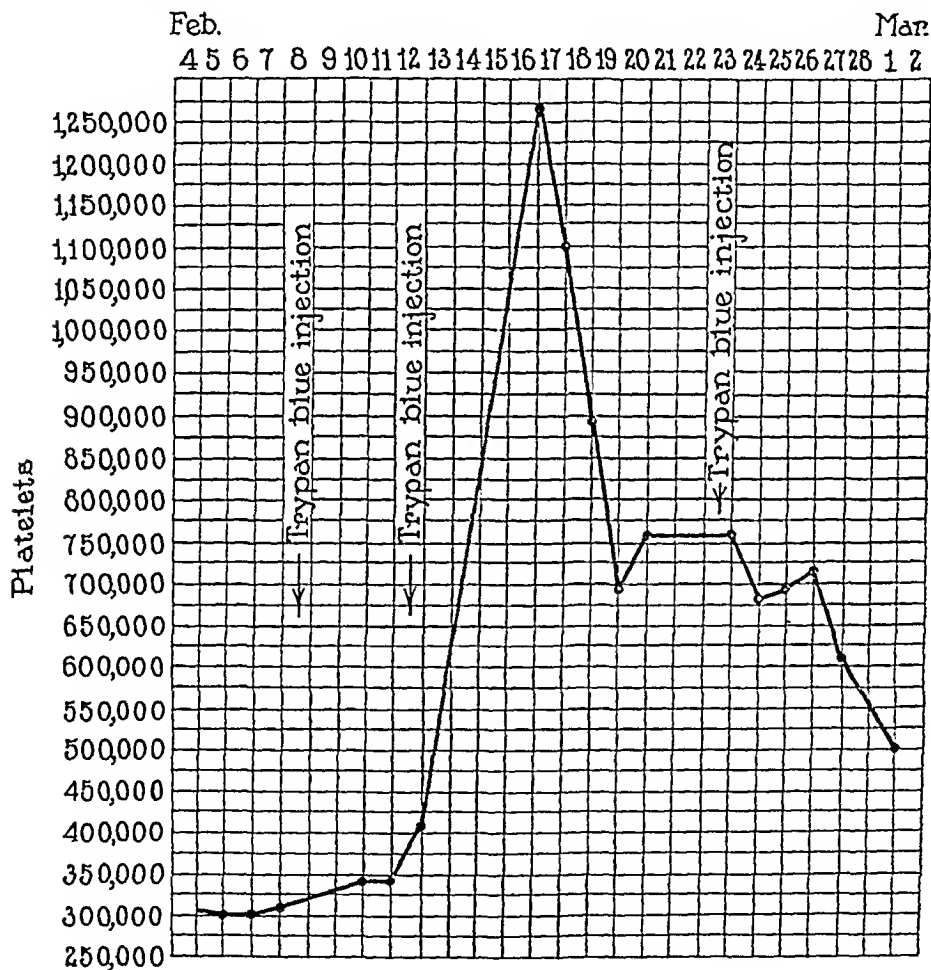


CHART 1. Graphic representation of the daily average platelet count of all the pigs used in this experiment.

DISCUSSION.

In analyzing the results of the experiment, it soon becomes apparent that many different conclusions might be drawn regarding the rela-

tionship of the reticulo-endothelial system to platelet destruction. Comparing the average counts before and after blockade, it would appear that the saturation interferes with the phagocytosis of platelets to a considerable degree. After a study of the graph however, the question as to what causes the sharp decline immediately suggests itself.

The later literature dealing with reticulo-endothelial system blockade by dyes and colloids supports the contentions of the earlier workers that the cells can be so overburdened as to have their activities paralyzed. The reticulo-endothelial cells have also been considered as the agents by which blood destruction is accomplished, chiefly because phagocytosis of red and white cells and platelets by the macrophages has been repeatedly noted. Such phagocytosis is especially prominent in pathological conditions. Asch found it prominent in the spleen in pernicious anemia. Seidelin reported it in malaria. Pool and Stillman recorded phagocytosis of platelets in the spleen in thrombocytopenic purpura. Enormous phagocytosis of erythrocytes occurs in birds poisoned by arsine. But even in normal healthy mammals such phagocytosis has long been recognized to occur in the spleen. Assuming then that the trypan blue completely blocked the reticulo-endothelial system and that phagocytosis is the only means by which platelets are destroyed, it would be expected that the platelets would continue to increase *ad infinitum* provided there was no inhibition of production.

It is doubtful however, whether all the reticulo-endothelial cells can be so completely stuffed as to be unable to function. As Rich says, "One might well expect the cells to be unable to live under such conditions." The blockade, and the inactivity, must be relative.

It is also doubtful whether phagocytosis is the only method of destroying platelets. In the case of the erythrocytes the evidence as reviewed by Rous decidedly favors the view that phagocytosis is not the only means for their destruction. Rous and Robertson have shown, for instance, that the cat, an animal with a very active hematopoietic system, has very few macrophages. Indeed, whether the cells which do undergo phagocytosis are primarily devoured by the macrophages, or whether they are first rendered inert and then picked up and metabolized merely as any other foreign bodies are, is still

open to question. It should be remembered that platelets are a very unstable quantity in the blood. The wide range of variation in the count under apparently normal conditions, the ease and rapidity with which they can be driven from the circulation by the injection of snake venom (Flexner), albumose (Gley), the rapidity with which they return to the circulation in severe cases of purpura hæmorrhagica after splenectomy, bespeak a very labile regulatory apparatus. And the side which deals with platelet production must not be overlooked.

The graph shows that after the initial marked rise and subsequent sharp decline, there is a steady diminution in the platelets despite the reinforcement of the blockade by another trypan blue injection. The possible explanations which suggest themselves are: (1) that the blockade is no longer as complete, despite the reinforcement, and that phagocytosis is now more active; (2) that in time some other mechanism assumes the added burden of platelet destruction as a compensatory reaction; (3) that the original trypan injection, while it blockaded the reticulo-endothelial system, sharply stimulated the platelet-producing mechanism to greater activity as manifested by the sudden and great increase in platelets, and subsequent injections failed because of acquired tolerance.

Achard and Aynaud report marked diminution in the platelet count after intravenous injection of electrargol. And yet many workers have used electrargol to block the reticulo-endothelial system. This is apparently a contradiction if blocking the system should cause a rise in the platelets. It would favor the idea that there is either some very active mechanism for platelet destruction beside phagocytosis, or that this particular agent caused inhibition of platelet production.

In another series of experiments undertaken to determine the effect of trypan blue blockade on the development of immune bodies, it was found that after the blockade had been established the injection of a small quantity of washed sheep red blood cells produced a fall from 1,000,000 platelets to 200,000 in 12 hours. This is also suggestive of other means of diminution in numbers besides phagocytosis.

It becomes quite evident that at this stage in our knowledge of the life cycle of the platelet it is far more important to accumulate data than to attempt to evolve hypotheses and construct theories, and that proper interpretation of the phenomena is dependent on the acquisition of more facts.

SUMMARY.

1. So called blockade of the reticulo-endothelial system results in a marked increase in the platelet count.

2. The count rapidly becomes very high, shows a sharp partial recoil and then a gradual diminution even though the blockade be continued.

3. The mechanism regulating the number of platelets is a complicated one.

4. Apparently phagocytosis by cells of the reticulo-endothelial system is not the only means by which blood platelets are destroyed.

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RELATION BETWEEN ORGAN WEIGHTS AND OBSCURE LESIONS IN APPARENTLY NORMAL RABBITS.

SECOND PAPER.

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An analysis of the results obtained from a study of organ weights of normal rabbits with reference to effects that might be produced by obscure lesions led to the conclusion (1) that while any abnormality might give rise to some change in the mass and mass relationships of organs, the magnitude of the change was of such an order that it was barely perceptible until the abnormality was sufficiently pronounced to be recognized by signs and symptoms of disease or by physical deterioration. In apparently healthy and vigorous rabbits it appeared that only those with extensive lesions were apt to show differences in the mean weights of organs that are of any material significance from a statistical point of view. It was thought, however, that even the small differences noted suggested a tendency to the occurrence of progressive changes in weight which bore some relation to the reaction of the animal to disease as indicated by the extent of the lesions present. In order to obtain further information concerning changes in organ weights that may occur in association with obscure disease conditions, an analysis of the results of a second series of weight determinations was undertaken. The object of this paper is to report the results of this investigation, which will include a consideration of the relation between organ weight and the activity of the lesions as well as the extent of the lesions found on postmortem examination.

Methods and Material.

The results to be reported are based on the examination of 295 male rabbits killed between July 1, 1924, and July 1, 1925, representing Series II of the normal rabbits included in the preceding paper of this group (2).

Net body weight.

2	80	237.2	229.25	128.3	452.0	50.30	33.90	21.20
A	16	237.5	230.00	166.0	344.0	46.20	31.20	19.45
I	64	237.2	229.50	128.3	452.0	51.40	34.70	21.66
3	51	240.2	241.00	117.0	374.0	53.50	36.10	22.27
A	19	225.8	230.00	117.0	351.0	54.90	37.03	24.31
I	32	248.8	246.00	156.0	374.0	50.80	34.30	20.41
4	26	223.9	220.00	171.0	357.5	34.60	23.30	15.40
A	13	207.5	199.00	171.0	313.0	25.40	17.10	12.24
I	13	240.4	234.00	177.5	357.5	38.10	25.70	15.80
II	295	1833.1	1800.0	1210	2660	273.00	184.10	14.89
1	138	1832.0	1805.0	1290	2630	280.03	188.80	15.29
2	80	1869.6	1842.5	1370	2545	258.92	174.64	13.84
A	16	1933.1	1910.0	1565	2483	295.60	199.40	15.29
I	64	1853.8	1835.0	1370	2545	227.95	153.75	12.29
3	51	1812.8	1715.0	1210	2660	273.70	184.60	15.09
A	19	1830.5	1790.0	1490	2350	236.10	159.30	12.89
I	32	1801.1	1702.5	1210	2660	293.40	197.90	16.29
4	26	1768.8	1735.0	1355	2500	276.70	186.60	15.60
A	13	1674.6	1580.0	1360	2135	215.50	145.40	12.86
I	13	1863.1	1815.0	1355	2500	285.20	192.40	15.30
II	295	5.31	5.22	3.30	9.02	0.93	0.627	17.51
1	138	5.305	5.40	3.30	9.02	0.908	0.612	17.09
2	80	5.30	5.19	3.45	8.14	0.902	0.608	17.01
A	16	5.57	5.71	4.00	8.14	1.05	0.71	18.85
I	64	5.24	5.15	3.45	8.05	0.874	0.59	16.67
3	51	5.34	5.30	3.47	7.43	0.896	0.604	16.77
A	19	5.39	5.30	3.55	7.40	0.943	0.636	17.49
I	32	5.31	5.31	3.47	7.43	0.867	0.585	16.32
4	26	5.34	5.29	3.38	7.63	1.13	0.76	21.16
A	13	5.32	5.05	3.79	7.50	1.066	0.719	20.00
I	13	5.36	5.45	3.38	7.63	1.19	0.803	22.21

Heart.
Actual.

TABLE I—Continued.

Organ.	Group No.	No. of animals.	Arithmetical mean.	Median.	Minimum.	Maximum.	Standard deviation.	Probable error.	Coefficient of variation.	
			gm.	gm.	gm.	gm.	gm.	±gm.	per cent	
Heart. Relative.	II	295	2.90	2.88	2.04	4.36	0.339	0.229	11.68	
	1	138	2.89	2.86	2.27	4.04	0.336	0.227	11.62	
	2	80	2.86	2.83	2.04	4.36	0.372	0.251	13.00	
	A	16	2.88	2.85	2.34	3.27	0.214	0.144	7.43	
	I	64	2.85	2.82	2.04	4.36	0.402	0.271	14.21	
	3	51	2.95	2.94	2.15	3.57	0.277	0.187	9.38	
	A	19	2.94	2.96	2.15	3.57	0.348	0.235	11.83	
	I	32	2.95	2.90	2.55	3.54	0.224	0.151	7.59	
	4	26	3.01	3.05	2.44	3.84	0.336	0.227	11.16	
	A	13	3.16	3.05	2.85	3.84	0.299	0.202	9.46	
	I	13	2.85	2.94	2.44	3.29	0.305	0.206	10.70	
	Liver. Actual	II	295	87.52	85.00	47.00	185.00	19.29	13.01	22.04
		1	138	87.05	85.00	47.00	153.00	18.46	12.45	21.19
2		80	88.26	86.00	57.00	152.00	19.21	12.96	21.90	
A		16	96.81	101.00	65.00	116.00	12.25	8.26	12.66	
I		64	86.12	80.00	57.00	152.00	20.10	13.56	23.34	
3		51	85.54	80.00	50.00	158.00	18.50	12.48	21.60	
A		19	84.10	85.00	55.00	117.00	17.60	11.87	20.92	
I		32	86.40	80.00	50.00	158.00	19.80	13.40	22.90	
4		26	94.96	92.00	63.00	185.00	23.07	15.56	24.29	
A		13	98.00	90.00	73.00	185.00	26.80	18.10	27.34	
I		13	91.92	92.00	63.00	120.00	18.70	12.60	20.34	
Relative.		II	295	48.47	46.70	25.80	117.00	12.10	8.16	24.96
		1	138	48.32	46.60	25.80	92.20	10.86	7.33	22.47

TABLE I—Continued.

Organ.	Group No.	No. of animals.	Arithmetical mean.	Median.	Minimum.	Maximum.	Standard deviation.	Probable error.	Coefficient of variation.
			gm.	gm.	gm.	gm.	± gm.	± gm.	per cent
Spleen. Actual.	II	295	1.065	0.985	0.160	5.050	0.549	0.370	51.54
	1	138	0.973	0.860	0.240	4.480	0.601	0.450	61.76
	2	80	1.060	1.025	0.320	2.370	0.448	0.302	42.26
	A	16	1.111	1.005	0.530	1.940	0.411	0.277	36.99
	I	64	1.048	1.013	0.320	2.370	0.457	0.308	43.60
	3	51	1.192	1.150	0.160	5.050	0.703	0.474	58.97
	A	19	1.230	1.150	0.160	5.050	0.969	0.654	78.78
	I	32	1.169	1.113	0.525	2.650	0.481	0.324	41.14
	4	26	1.315	1.192	0.600	2.685	0.533	0.359	40.53
	A	13	1.325	1.220	0.600	2.135	0.420	0.283	31.69
	I	13	1.307	1.185	0.660	2.685	0.620	0.418	47.14
Relative.	II	295	0.587	0.528	0.107	2.930	0.326	0.220	55.53
	1	138	0.520	0.463	0.140	2.140	0.3004	0.203	57.76
	2	80	0.572	0.549	0.168	1.480	0.246	0.166	43.01
	A	16	0.582	0.574	0.262	0.964	0.213	0.144	36.60
	I	64	0.570	0.539	0.168	1.480	0.254	0.171	44.50
	3	51	0.670	0.631	0.107	2.930	0.414	0.279	61.79
	A	19	0.679	0.545	0.107	2.930	0.568	0.383	83.65
	I	32	0.665	0.653	0.287	1.620	0.292	0.197	43.90
	4	26	0.751	0.722	0.355	1.440	0.302	0.204	40.21
	A	13	0.785	0.722	0.441	1.282	0.196	0.132	24.96
	I	13	0.717	0.544	0.355	1.440	0.352	0.237	49.09
Thymus. Actual.	II	295	2.365	2.250	0.37	6.30	0.842	0.568	35.67
	1	135	2.450	2.350	0.83	6.30	0.848	0.572	34.61

TABLE I—Continued.

Organ.	Group No.	No. of animals.	Arithmetical mean.		Median.	Minimum.		Maximum.	Standard deviation.		Probable error.	Coefficient of variation.	
			gm.	gm.		gm.	gm.		± gm.	per cent			
Testicles. Relative.	II	294	2.45	2.42	0.562	4.220	0.650	0.440	26.53				
	1	137	2.37	2.37	0.902	4.160	0.640	0.432	27.00				
	2	80	2.54	2.50	0.990	4.220	0.610	0.411	24.01				
	A	16	2.47	2.53	1.260	3.335	0.440	0.300	17.80				
	I	64	2.57	2.48	0.990	4.220	0.639	0.431	24.80				
	3	51	2.47	2.43	0.562	4.170	0.680	0.460	27.53				
	A	19	2.54	2.28	1.740	4.170	0.540	0.360	21.25				
	I	32	2.43	2.47	0.562	3.910	0.750	0.500	30.86				
	4	26	2.45	2.46	1.250	4.220	0.650	0.440	26.53				
	A	13	2.57	2.73	1.250	4.220	0.770	0.520	29.96				
	I	13	2.32	2.44	1.380	3.020	0.480	0.324	20.69				
	Brain. Actual.	II	295	9.11	9.20	5.80	11.33	0.687	0.463	7.54			
		1	138	9.08	9.15	5.80	11.30	0.692	0.467	7.62			
2		80	9.19	9.30	7.43	10.80	0.630	0.425	6.80				
A		16	9.07	9.06	7.57	10.80	0.740	0.500	8.16				
I		64	9.22	9.32	7.43	10.63	0.586	0.395	6.35				
3		51	9.16	9.25	7.94	11.33	0.604	0.407	6.59				
A		19	9.37	9.35	8.10	11.33	0.649	0.437	6.93				
I		32	9.04	9.18	7.94	10.45	0.538	0.363	5.95				
4		26	8.88	8.90	8.00	9.82	0.448	0.302	5.04				
A		13	8.77	8.85	8.00	9.60	0.420	0.283	4.79				
I		13	8.99	8.95	8.00	9.82	0.478	0.322	5.32				
Relative.		II	295	5.06	5.01	3.45	7.08	0.678	0.457	13.39			
		1	138	5.05	4.98	3.45	7.08	0.6501	0.438	12.87			
	2	80	5.03	4.99	3.67	6.84	0.668	0.451	13.28				
	A	16	4.81	4.65	3.77	5.76	0.625	0.422	12.99				
	I	64	5.09	5.04	3.67	6.84	0.670	0.452	13.16				

MONOGRAPHS—Continued.

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TABLE I—Continued.

Organ.	Group No.	No. of animals.	Arithmetical mean.	Median.	Minimum.	Maximum.	Standard deviation.	Probable error.	Coefficient of variation.
			gm.	gm.	gm.	gm.	±gm.	±gm.	per cent
Parathyroids. Actual.	II	292	0.0132	0.012	0.003	0.035	0.00551	0.00372.	41.74
	1	137	0.01299	0.012	0.004	0.030	0.00502	0.00339	38.60
	2	79	0.01325	0.012	0.003	0.035	0.005403	0.00364	40.77
	A	16	0.01337	0.012	0.006	0.025	0.00478	0.00322	35.75
	I	63	0.01322	0.012	0.003	0.035	0.00555	0.00374	41.98
	3	50	0.01384	0.012	0.005	0.027	0.00522	0.00352	37.71
	A	19	0.01442	0.012	0.010	0.025	0.00515	0.00347	35.71
	I	31	0.01348	0.012	0.005	0.027	0.00524	0.00353	38.81
	4	26	0.01388	0.013	0.006	0.033	0.00616	0.00415	44.38
	A	13	0.01353	0.012	0.006	0.033	0.00659	0.00444	48.70
	I	13	0.01424	0.013	0.007	0.025	0.00565	0.00381	39.67
Relative.	II	292	0.00730	0.00667	0.00178	0.01850	0.00289	0.00195	39.60
	1	137	0.007094	0.00639	0.00266	0.01366	0.00269	0.001814	37.94
	2	79	0.00722	0.00656	0.00178	0.01850	0.00306	0.00206	42.38
	A	16	0.00696	0.00598	0.00383	0.01467	0.00265	0.00179	38.07
	I	63	0.00729	0.00667	0.00178	0.01850	0.00315	0.00212	43.20
	3	50	0.0077	0.00639	0.00306	0.01515	0.00295	0.00199	38.31
	A	19	0.0079	0.00725	0.00482	0.01501	0.0027	0.00182	34.17
	I	31	0.00757	0.00636	0.00306	0.01515	0.00309	0.00208	40.81
	4	26	0.00785	0.00758	0.00382	0.01545	0.00311	0.00210	39.61
	A	13	0.00793	0.00735	0.00382	0.01545	0.00301	0.00203	37.95
	I	13	0.00776	0.00823	0.00409	0.01454	0.00319	0.00215	41.10
Hypophysis. Actual.	II	294	0.0283	0.028	0.015	0.050	0.00553	0.00373	19.55
	1	138	0.028	0.027	0.015	0.050	0.00623	0.0042	22.24

Thyroid.
Actual.

Relative

3	51	5.14	5.22	3.66	7.02	0.648	0.437	12.60
A	19	5.17	5.13	4.20	6.21	0.510	0.340	9.86
I	32	5.12	5.27	3.66	7.02	0.716	0.483	13.98
4	26	5.11	4.99	3.93	6.69	0.679	0.455	7.52
A	13	5.31	5.32	4.33	6.69	0.630	0.425	11.86
I	13	4.91	4.85	3.93	6.60	0.620	0.418	12.62
II	295	0.2356	0.210	0.085	1.730	0.1326	0.08944	56.28
1	138	0.2175	0.200	0.090	0.545	0.08285	0.05588	38.09
2	80	0.2603	0.210	0.100	1.730	0.2061	0.13901	79.17
	(79)	(0.24170)		(0.090)	(0.935)	(0.1238)	(0.0835)	(51.22)
A	16	0.2183	0.2175	0.105	0.325	0.0602	0.0406	27.57
I	64	0.2709	0.209	0.100	1.730	0.2274	0.15338	83.94
	(63)	(0.2477)		(0.100)	(0.935)	(0.1349)	(0.09099)	(54.46)
3	51	0.23645	0.220	0.135	0.480	0.0824	0.05558	34.82
A	19	0.23426	0.212	0.135	0.512	0.0922	0.06219	39.36
I	32	0.23775	0.2225	0.140	0.480	0.07606	0.05133	31.98
4	26	0.2546	0.250	0.085	0.775	0.12202	0.0823	47.92
A	13	0.2406	0.245	0.155	0.340	0.05209	0.03513	21.65
I	13	0.2686	0.250	0.085	0.775	0.16310	0.11001	60.72
II	295	0.13030	0.1149	0.0481	1.230	0.08119	0.05476	62.31
I	138	0.1199	0.1084	0.0569	0.283	0.04611	0.03111	38.46
2	80	0.14403	0.11435	0.0562	1.230	0.1371	0.09247	95.21
	(79)	(0.13030)		(0.0562)	(0.392)	(0.0626)	(0.0422)	(48.04)
A	16	0.1131	0.10745	0.0686	0.1968	0.0291	0.01963	25.72
I	64	0.1518	0.1174	0.0562	1.230	0.1515	0.10219	99.80
	(63)	(0.1347)		(0.0562)	(0.392)	(0.0678)	(0.0457)	(50.33)
3	51	0.13069	0.1215	0.0798	0.3135	0.0415	0.02799	31.75
A	19	0.12685	0.1215	0.0798	0.218	0.0398	0.02685	31.36
I	32	0.13297	0.12025	0.0843	0.3135	0.0423	0.02853	31.80
4	26	0.1428	0.1383	0.0481	0.310	0.0517	0.03487	36.20
A	13	0.1492	0.141	0.0994	0.2092	0.0369	0.02489	24.73
I	13	0.1397	0.136	0.0481	0.3100	0.0631	0.04256	45.16

Relative.

Suprenenals.
Actual.

2	A	80	0.0295	0.0295	0.015	0.040	0.00535	0.00361	18.11
	I	16	0.0311	0.030	0.022	0.050	0.00654	0.00441	21.01
3		64	0.02914	0.0285	0.015	0.040	0.00492	0.00235	16.88
	A	50	0.02782	0.028	0.020	0.040	0.00418	0.00282	15.02
	I	19	0.02742	0.028	0.022	0.033	0.00331	0.00223	12.08
4		31	0.02806	0.028	0.020	0.040	0.00461	0.00311	16.42
	A	26	0.02715	0.0255	0.020	0.035	0.00383	0.00258	14.10
	I	13	0.02707	0.027	0.020	0.035	0.00417	0.00281	15.40
		13	0.02723	0.025	0.023	0.035	0.00352	0.00237	12.92
II		294	0.01566	0.01535	0.0078	0.0352	0.00322	0.00217	20.56
I		138	0.0155	0.01505	0.0078	0.0352	0.00351	0.00237	22.64
2		80	0.01606	0.01577	0.00944	0.0247	0.00294	0.00198	18.30
	A	16	0.0164	0.01605	0.01054	0.0255	0.00479	0.00323	29.20
	I	64	0.01598	0.01587	0.00944	0.0247	0.00274	0.00184	17.14
3		50	0.01557	0.01591	0.01005	0.0244	0.00292	0.00197	18.75
	A	19	0.01522	0.01565	0.01005	0.01942	0.00262	0.00177	17.21
	I	31	0.01579	0.01591	0.01096	0.0244	0.00307	0.00207	19.43
4		26	0.01567	0.01614	0.00960	0.0235	0.00304	0.00205	19.40
	A	13	0.01640	0.01655	0.01143	0.0235	0.00318	0.00215	19.39
	I	13	0.01495	0.01580	0.0096	0.01845	0.00389	0.00262	26.02
II		295	0.3802	0.350	0.138	1.050	0.1646	0.11102	43.29
I		138	0.3571	0.3335	0.145	0.987	0.14206	0.09582	39.78
2		80	0.39015	0.350	0.160	0.885	0.1612	0.10873	41.31
	A	16	0.40593	0.3875	0.240	0.770	0.1416	0.09551	34.93
	I	64	0.3862	0.3375	0.160	0.885	0.1655	0.11163	42.85
3		51	0.40245	0.360	0.165	0.900	0.1717	0.11581	42.65
	A	19	0.37157	0.330	0.165	0.900	0.1775	0.11972	47.76
	I	32	0.42078	0.3775	0.200	0.720	0.1683	0.11352	39.99
4		26	0.4284	0.3575	0.138	1.050	0.2388	0.16107	55.74
	A	13	0.35561	0.310	0.138	0.760	0.1736	0.11709	48.81
	I	13	0.50115	0.400	0.200	1.050	0.3876	0.26144	77.34

Popliteal lymph nodes.
Actual.

2	A	78	0.00842	0.00825	0.00263	0.0185	0.00272	0.00183	32.30
	I	16	0.0084	0.00872	0.00321	0.0176	0.00339	0.00229	40.35
3		62	0.00843	0.00815	0.00263	0.0185	0.00252	0.0017	29.89
	A	51	0.00889	0.00883	0.00228	0.0247	0.0035	0.00236	39.37
	I	19	0.00867	0.00904	0.00228	0.012	0.00207	0.0014	23.87
4		32	0.00903	0.00840	0.00489	0.0247	0.00411	0.00277	45.52
	A	26	0.00786	0.00821	0.00302	0.0128	0.00267	0.0018	33.96
	I	13	0.00804	0.00822	0.00307	0.01225	0.00215	0.00145	26.74
	I	13	0.00768	0.00661	0.00302	0.0128	0.00311	0.00209	40.44
II		294	0.25612	0.245	0.090	0.570	0.0769	0.05187	30.02
1		137	0.26434	0.250	0.100	0.570	0.0751	0.05065	28.41
2		80	0.24731	0.2375	0.100	0.465	0.0781	0.0527	31.58
	A	16	0.26218	0.250	0.135	0.440	0.0802	0.05409	30.58
	I	64	0.24359	0.235	0.100	0.465	0.0772	0.05207	31.69
3		51	0.23974	0.235	0.090	0.450	0.0737	0.0497	30.74
	A	19	0.24063	0.237	0.090	0.420	0.0844	0.05693	35.07
	I	32	0.23921	0.2225	0.135	0.450	0.0667	0.04499	27.88
4		26	0.2713	0.2725	0.145	0.450	0.0793	0.0535	29.22
	A	13	0.26946	0.270	0.155	0.450	0.0879	0.0593	32.62
	I	13	0.27308	0.290	0.145	0.400	0.0697	0.04701	25.52
II		294	0.1425	0.1388	0.050	0.3335	0.04415	0.02978	30.98
1		137	0.14653	0.139	0.050	0.3335	0.04511	0.03043	30.78
2		80	0.13254	0.136	0.0563	0.2405	0.0382	0.02577	28.80
	A	16	0.13523	0.13835	0.0782	0.1965	0.0348	0.02347	25.70
	I	64	0.13186	0.13295	0.0563	0.2405	0.0396	0.0267	30.03
3		51	0.13437	0.1375	0.0524	0.275	0.0431	0.02908	32.06
	A	19	0.13105	0.1415	0.0524	0.1965	0.0414	0.02792	31.57
	I	32	0.13635	0.137	0.0718	0.275	0.0441	0.02975	32.33
4		26	0.1522	0.15325	0.0592	0.2645	0.0505	0.03406	33.18
	A	13	0.16302	0.1442	0.082	0.2645	0.0552	0.0372	33.86
	I	13	0.14136	0.153	0.0592	0.2035	0.0429	0.02894	30.34

Relative.

TABLE I—*Concluded.*

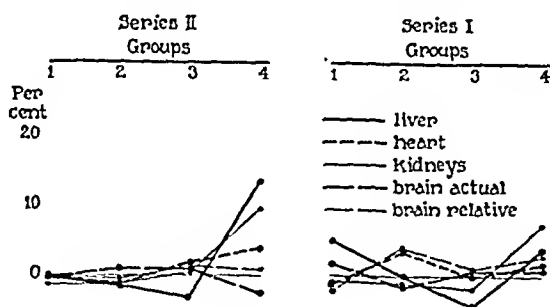
Organ.	Group No.	No. of animals.	Arithmetical mean.		Median.	Minimum.		Maximum.	Standard deviation.		Probable error.	Coefficient of variation.
			gm.	gm.		gm.	gm.		± gm.	± gm.		
Mesenteric lymph nodes. Relative.	II	293	1.915	1.830	0.67	6.91	0.642	0.433	33.52			
	1	136	1.951	1.870	0.67	6.91	0.715	0.482	37.33			
	2	80	1.856	1.815	0.902	3.65	0.589	0.397	31.73			
	A	16	1.654	1.618	1.105	2.38	0.306	0.206	18.50			
	I	64	1.907	1.833	0.902	3.65	0.628	0.424	32.93			
	3	51	1.911	1.855	0.806	3.44	0.547	0.369	28.62			
	A	19	1.758	1.635	0.806	3.09	0.527	0.355	29.97			
	I	32	2.002	2.018	1.155	3.44	0.541	0.365	27.02			
	4	26	1.834	1.653	0.936	2.88	0.508	0.343	27.69			
	A	13	1.953	1.830	1.30	2.77	0.357	0.241	18.27			
	I	13	1.714	1.64	0.936	2.88	0.532	0.359	31.03			

2	A	78	0.16098	0.1375	0.070	0.660	0.09360	0.06313	58.14
		14	0.22785	0.185	0.125	0.560	0.11318	0.07634	49.67
3	I	64	0.14635	0.1275	0.070	0.660	0.05454	0.03679	37.26
		49	0.16283	0.140	0.075	0.360	0.0648	0.04371	39.80
4	A	17	0.17494	0.170	0.100	0.255	0.0524	0.03534	29.95
		32	0.1564	0.130	0.075	0.360	0.07001	0.04722	44.76
4	I	26	0.1554	0.1275	0.060	0.560	0.1113	0.07507	71.62
		13	0.190	0.135	0.090	0.560	0.1468	0.09902	77.26
II	I	13	0.1207	0.125	0.060	0.175	0.0276	0.0186	22.86
II	I	239	0.0858	0.0738	0.0307	0.295	0.0414	0.0279	48.25
		136	0.08311	0.743	0.0319	0.221	0.03202	0.0216	38.52
2	A	78	0.08566	0.0738	0.0398	0.295	0.04504	0.03038	52.57
		14	0.1163	0.0931	0.0656	0.252	0.05402	0.03643	46.44
3	I	64	0.07896	0.07075	0.0398	0.295	0.0398	0.02685	50.40
		49	0.09084	0.0767	0.0456	0.227	0.0379	0.02556	41.72
4	A	17	0.0959	0.097	0.0456	0.165	0.0336	0.02266	35.03
		32	0.08815	0.0718	0.0456	0.227	0.0401	0.02705	45.46
4	I	26	0.0906	0.0704	0.0307	0.361	0.0693	0.04674	76.49
		13	0.11533	0.076	0.0574	0.361	0.0901	0.06077	78.12
II	I	13	0.06589	0.0652	0.0307	0.0992	0.0162	0.01093	24.58
II	I	293	3.460	3.340	1.07	11.47	1.133	0.764	32.75
		136	3.515	3.335	1.07	11.47	1.260	0.850	35.84
2	A	80	3.456	3.375	2.00	6.75	0.953	0.643	27.57
		16	3.150	3.170	2.20	4.27	0.533	0.360	16.92
3	I	64	3.532	3.425	2.00	6.75	1.020	0.688	28.87
		51	3.450	3.350	1.20	6.20	1.060	0.710	30.70
4	A	19	3.250	3.000	1.20	6.17	1.160	0.780	35.60
		32	3.560	3.500	2.00	6.20	0.987	0.666	27.72
4	I	26	3.195	3.080	1.58	5.60	0.863	0.582	27.01
		13	3.215	3.100	2.27	4.35	0.601	0.405	18.69
II	I	13	3.176	2.840	1.58	5.60	1.068	0.720	33.62

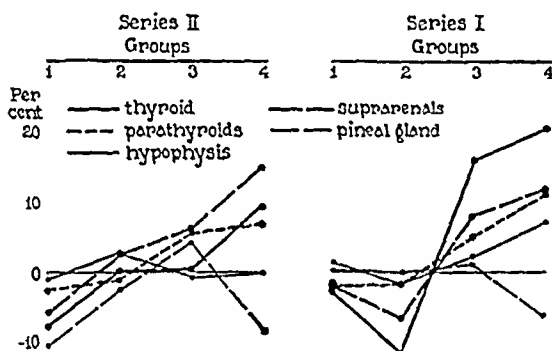
Mesenteric lymph nodes.

Actual.

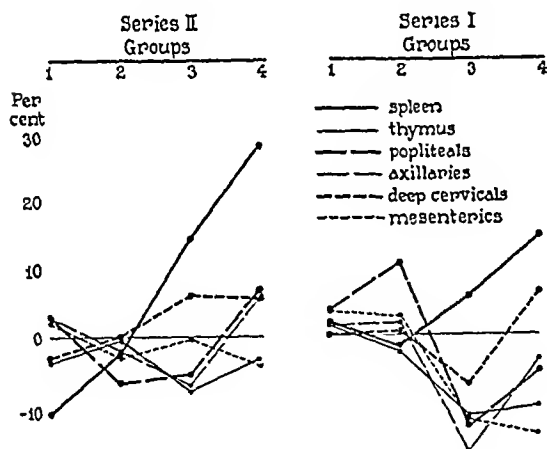
due to an increase in the number of animals with slight or moderate lesions; marked lesions were less frequent. The comparative per-



TEXT-FIG. 1.



TEXT-FIG. 2.



TEXT-FIG. 3.

TEXT-FIGS. 1 to 3. Percentage deviation of mean organ weight from the mean weight of the series.

The method employed in conducting the investigation and the classification of animals with reference to the extent of the lesions found at autopsy are described in detail elsewhere (1, 3). The same classification of material was followed in the separation of animals with active from those with inactive lesions. In this case, a primary division of animals was made according to the extent of the lesions found at autopsy and each of these groups was further divided on the basis of the activity of the lesions present. For this purpose, all animals presenting lesions that were healed or in an advanced stage of healing or resolution were classed as inactive, while those with any lesions which appeared to be progressing or showed no indication of healing were classed as active. We thus have one group of animals that showed no gross lesions at autopsy and three other groups with lesions that were slight, moderate, or marked; these three groups are subdivided into those with active and those with inactive lesions.

For convenience, the results of the statistical analysis of data are summarized in Table I which contains values for actual weights and for weights per kilo of net body weight (relative weight) as defined in a previous paper (3). Each set of figures gives the values obtained for the entire group of animals (295) and corresponding values for those with no lesions and those with active and with inactive lesions that were slight, moderate, or marked, in the order mentioned. These subgroups are designated by the arabic numerals 1, 2, 3, and 4; and the active and inactive divisions of each by the letters *A* and *I* respectively. The figures given in brackets for the thyroid in subgroup 2I, actual and relative, are values obtained by the omission of the weight of one large goiter. In Text-figs. 1 to 3, mean organ weights of the four subgroups of Series II are compared with corresponding values for the subgroups of Series I on the basis of the percentage deviation of the mean weight of a given organ from the mean weight for that organ for Series I or II as the case may be. Graphs of the coefficients of variation are given in Text-figs. 4 to 6.

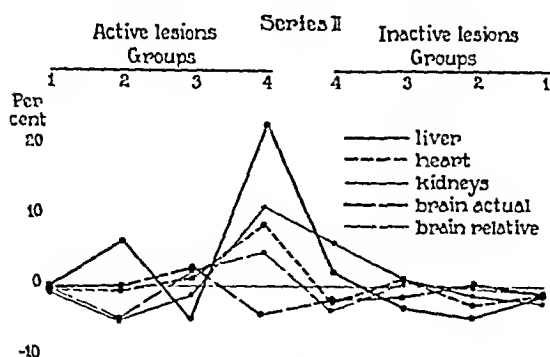
Mean organ weights and coefficients of variation for animals with active and with inactive lesions are compared graphically in Text-figs. 7 to 15. In this case, the values are arranged in the form of a progressive series, reading from left to right. The first half of each text-figure gives the results for animals with active lesions arranged in the order of increasing abnormality, while the second half gives corresponding values for animals with inactive lesions arranged in the order of decreasing abnormality. The first and last values are those for strictly normal animals (no lesions found at autopsy).

RESULTS.

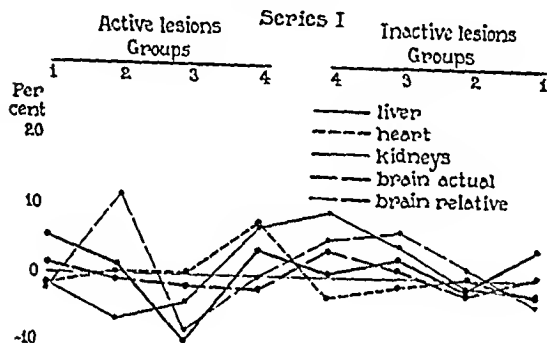
The results are summarized in Table I and Text-figs. 1 to 15.

DISCUSSION AND CONCLUSIONS.

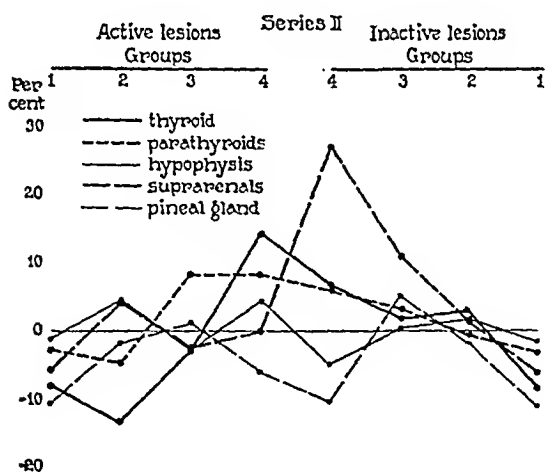
The prevalence of gross lesions among the animals of the present series (Series II) was somewhat greater than in the first (Series I)



TEXT-FIG. 7.

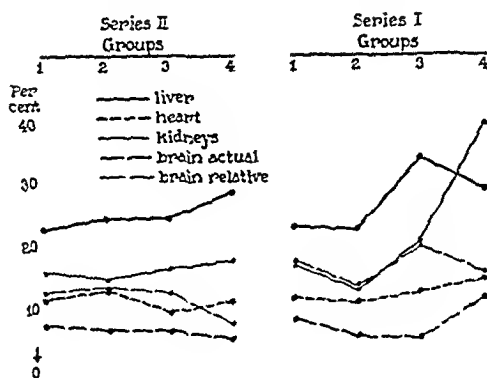


TEXT-FIG. 8.

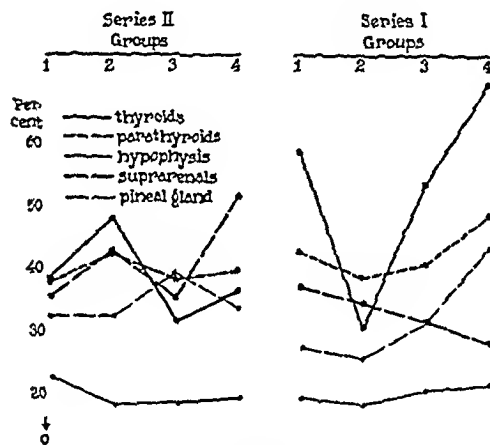


TEXT-FIG. 9.

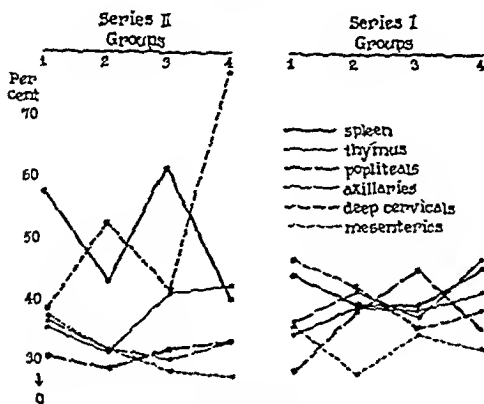
TEXT-FIGS. 7 to 12. Percentage deviation of mean organ weights from the mean for all animals of the series.



TEXT-FIG. 4.

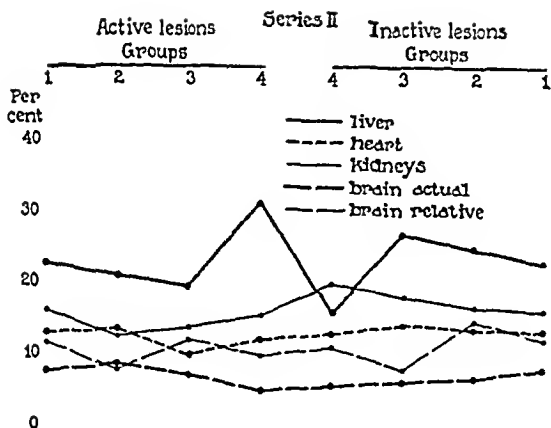


TEXT-FIG. 5.

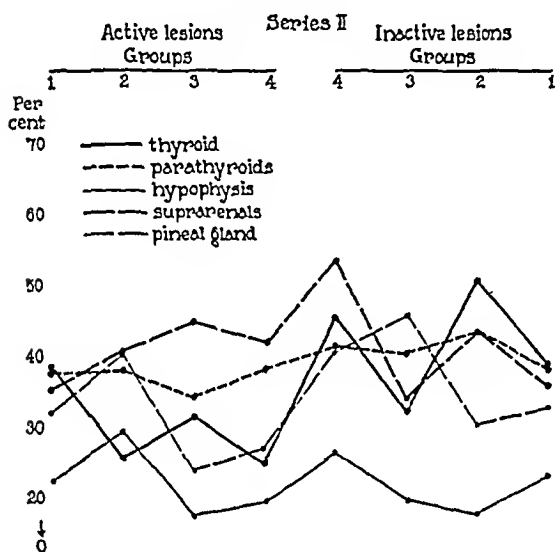


TEXT-FIG. 6.

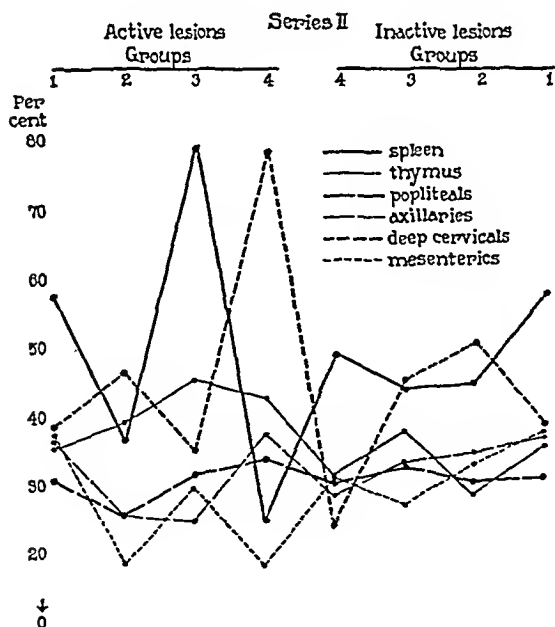
TEXT-FIGS. 4 to 6. Coefficients of variation for organ weight.



TEXT-FIG. 13.

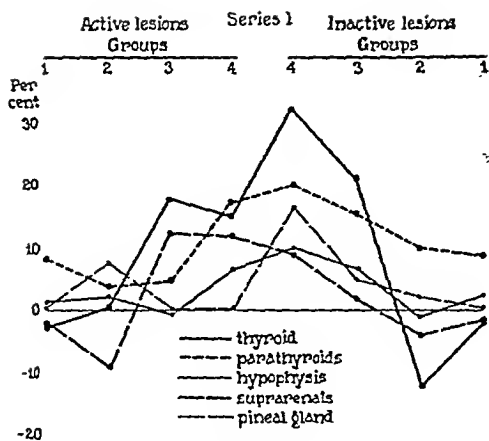


TEXT-FIG. 14.

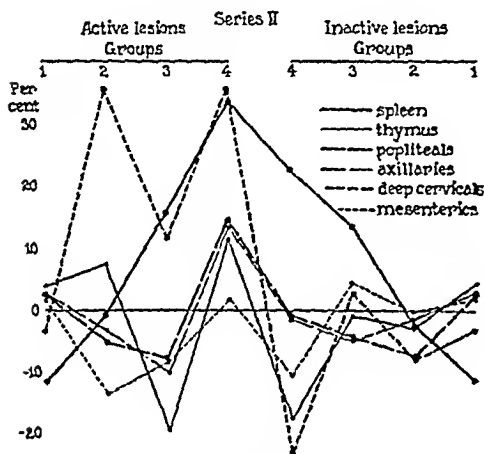


TEXT-FIG. 15.

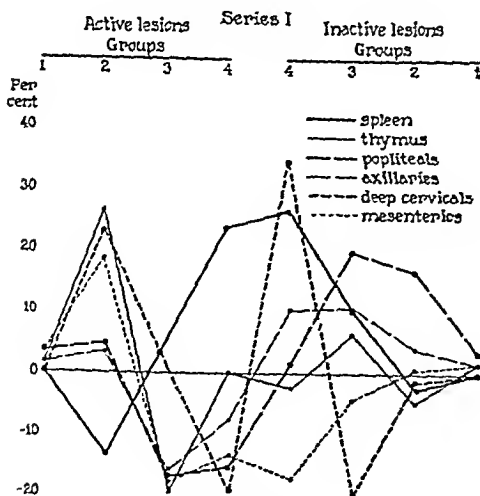
TEXT-FIGS. 13 to 15. Coefficients of variation for organ weight.



TEXT-FIG. 10.



TEXT-FIG. 11.



TEXT-FIG. 12.

weights (coefficients of variation). The graphs (Text-figs. 4 to 6) indicate a closer agreement than the tabulated results, which again emphasizes the fact that the nature of the effect is more constant than the magnitude.

With reference to the apparent discrepancies between the results obtained for Series I and II, attention may be called to the fact that there were certain important differences in the material which un-

TABLE II.
Distribution of Maximum and Minimum Mean Weights and Coefficients of Variation According to Series and Groups.

Organ.	Mean weight.				Coefficient.			
	Maximum.		Minimum.		Maximum.		Minimum.	
	I	II	I	II	I	II	I	II
Thyroid.....	4	4	2	1	4	2	2	3
Hypophysis.....	4	2	2	1	4	1	2	2
Suprarenals.....	4	4	2	1	4	2	3	3
Parathyroids.....	4	4	1	1	4	2	2	1
Pineal gland.....	3	3	4	1	3	4	2	2
Popliteal lymph nodes.....	2	4	3	2	3	4	1	2
Axillary " ".....	2	4	3	3	4	1	1	3
Mesenteric " ".....	1	1	4	4	1	1	2	4
Thymus.....	1	1	3	3	4	4	1	2
Heart.....	2	4	1	2	4	2	2	3
Brain.....	2	3	1	2	3	2	2	4
Liver.....	1	4	3	3	3	4	2	1
Kidneys.....	4	4	3	1,2	4	4	2	2
Spleen.....	4	4	2	1	4	3	3	4
Deep cervical lymph nodes.....	4	3	3	1	1	4	3	1

doubtedly affected the results to some extent. In the first place, the nature and extent of the lesions presented and the distribution of different types of disease among the various subgroups were not the same in the two cases. Moreover, it will be noted that the normal values for Series II (Group 1) are lower on the whole than the corresponding values for Series I, which indicates the existence of a different state of organ balance due to the action of factors other than disease.

Differences of this kind may have some influence on the apparent effect of slight, moderate, or marked lesions on organ weight. For

centage distribution of animals among the four subgroups and the combined incidence (645 rabbits) were as follows:

Group.	Series I.	Series II.	Total.
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
1	59.1	46.8	53.49
2	20.3	27.1	23.41
3	10.3	17.3	13.49
4	10.3	8.8	9.61

The incidence of gross lesions in the entire group of 645 apparently healthy stock rabbits was, therefore, approximately 50 per cent (46.51 per cent); the extent of the lesions varied within comparatively narrow limits and only a small proportion of the animals (9.61 per cent), or about 1 in 10, showed extensive lesions, either active or healed.

The apparent effects of the lesions, or disease processes, on organ weights, as measured by mean weights and the tendency to variation, were of the same general order as in the first series of animals studied, and while the values obtained were not exactly the same in the two cases the agreement is sufficiently close to indicate that the results are significant. By reference to Text-figs. 1 to 3, it will be seen that the direction of the effect of marked lesions on mean organ weights and coefficients of variation as compared with normal animals (Groups 1 and 4) is usually the same. If the weight is increased in one case it is usually increased in the other or if it is diminished in one case it is diminished in the other, but the magnitude of the change differs and there is greater variation in the apparent effects of slight or moderate lesions. The agreement or disagreement between the two sets of animals (Series I and II) may be brought out by a tabulation of the distribution of maximum and minimum mean weights (per kilo of net body weight) and of coefficients of variation according to groups (Table II).

Table II shows that, with very few exceptions, the presence of obscure lesions was associated with an increase in the mean weight of organs per kilo of body weight; the two striking exceptions are the thymus and the mesenteric lymph nodes. There is less certainty concerning the relation of obscure lesions to the uniformity of organ

plotted. The results show essentially the same departure from and return to normal as was found in the case of rabbits presenting clinical manifestations of disease (4).

The curves for the coefficients of variation are more irregular and are difficult to interpret. If we disregard point to point fluctuations, however, and consider only the general direction of the change from normal to the most marked condition of active disease and from the most marked inactive lesions back to normal (Groups 1 to 4 and 4 to 1) it will be seen that there are distinct differences between the variability of weight for the two major groups of animals and that, in general, the direction of the curves bears a relation to the curves for mean weight. As an example of this condition, reference to Text-figs. 11 and 15 will show that the weight of the spleen increases progressively in animals with active lesions and decreases in the same manner in animals with inactive lesions; but the coefficients of variations are extremely irregular owing chiefly to the value given by the animals of Group 3 (active). Still, the general direction of the curve is downward from Group 1 to Group 4 and is upward from this point to Group 1 (reading from left to right), or is just the reverse of the weight curve. This indicates that the increase in mean weight was accompanied by an increase in the uniformity of weights for individual organs (except in Group 3) and that as the weight of the spleen diminished or returned to normal there was also a reduction in the uniformity of weights. Other organs show analogous relationships between the two sets of curves. Moreover, it will be seen that in some cases the curves for coefficients of active and of inactive lesions agree with those for the coefficients of Series I or II and that some of the discrepancies between the curves of these two series may be accounted for by the activity of the lesions of different groups of animals.

The results of this study of organ weights and obscure disease conditions in normal rabbits may be summarized briefly by saying that it furnishes conclusive evidence of a close relation between physical constitution, as represented by organ weight, and susceptibility to disease, as expressed by the extent and the activity of the lesions found post mortem. The evidence available indicates that disease, even in its mildest form, is capable of affecting the weights

example, it will be seen by reference to Text-fig. 2 that in the case of animals of Series I with slight lesions (Group 2), the weights of certain organs diminished to a point comparable to that of the animals in Group 1 of Series II before the upward movement took place. It will also be seen that the decided reduction in the weights of the thyroid and suprarenals of Series I (Text-fig. 2) was offset by a corresponding increase in the weights of lymph nodes (Text-fig. 3) and that the reduction and subsequent increase in the weights of the lymph nodes in animals with more extensive lesions was associated with a greater and corresponding increase in the weights of thyroids and suprarenals.

Such differences as these suggest that the apparent effect of a given stimulus may vary, depending upon the state of the organ (or organism) during the time the stimulus is operative or upon the effect produced by other stimuli. When we take into account even the known differences between the corresponding groups of animals of the two series, the extent of the agreement is surprising, especially so in view of the comparatively small absolute difference in the magnitude of the values with which we are dealing. It is obvious, however, that, as even the direction of the change is subject to some variation, less significance can be attached to the magnitude of the effect produced.

A comparison of results on the basis of the activity of the lesions furnishes further confirmation of the occurrence of alterations in organ weight in response to the action of disease processes which produce no clinical manifestations of disease. In addition, it appears that there are some differences between the effects of lesions that are active and those that are inactive as defined above. In Text-figs. 7 to 15, the results have been arranged so as to convey the idea of a progressive increase in the extent of the disease (reading from left to right) with a change from an active to an inactive state and a progressive reduction in the extent of the lesions with a return to normal. This is, of course, only a schematic representation with a wide gap at the point of transition from active to inactive lesions.

Here again the general direction of the curves from Group 1 to Group 4 and from 4 to 1 is of more importance than the magnitude of the difference between any two points. The curves for mean weight, in general, bear a relation to the extent and state of the lesions as

of organs that are not directly involved by the disease process and that the effect produced bears a relation to both the extent and the activity of the lesions present. There is some evidence that the converse may be true also; namely, that the occurrence and subsequent course of disease are influenced in some measure by the state of organ balance and the ability of the animal organism to adjust itself to meet the demands of disease-producing agencies.

SUMMARY.

A study of organ weights was made on a group of 295 normal rabbits for the purpose of determining whether any relation could be detected between the weights of organs and the extent and activity of the lesions found post mortem.

The results obtained seemed to indicate that disease, even in its mildest form, is capable of affecting the weights of organs that are not directly involved by the disease process and that the effect produced bears a relation to both the extent and the activity of the lesions present. There was also some evidence that the converse might be true; namely, that the occurrence and subsequent course of disease may be influenced in some measure by the state of organ balance and the ability of the animal organism to adjust itself to meet the demands of disease-producing agencies.

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could determine no serological differences between these organisms. Goldzieher and Neuber (20), on the other hand, obtained fixation reactions with *B. rhinoscleromatis*, using serum from patients suffering from rhinoscleroma and from rabbits immunized to the rhinoscleroma bacillus, but they were not successful when Friedländer's bacillus was used as antigen with the same sera. Babes (21) produced in rabbits an agglutinating serum of low titer against a strain of *B. rhinoscleromatis*. Sdrawosmysloff (22) found no complement-fixing antibodies in the serum of a rhinoscleroma patient.

Schmidt (23), using the serum of a patient convalescent from Friedländer infection of the lung, and the organism isolated from the sputum and from the lung by direct puncture, observed an "amorphous" agglutination. In this instance, no immune properties were demonstrable until after the 1st month of illness, when agglutination, bacteriolysis, and thread reaction were observed. Wolf (24) isolated a strain of Friedländer's bacillus from the urine of a patient whose serum agglutinated equally well the homologous strains and several stock laboratory ones in dilutions of 1:2000 and 1:5000.

Porges attributed the difficulty of immunization with *encapsulatus* strains to the presence of capsules which he thought interfered also with agglutination. Consequently he devised a method by which the capsular material was hydrolyzed by heating suspensions of the bacteria in weak acid. With von Eisler (25) he showed that after destruction of the capsule immune sera could be obtained and that agglutination was facilitated in this way. Streit (26) and Beham (27) took exception to the method because suspensions so treated were apt to clump spontaneously. They effected decapsulation by cultural methods, and obtained agglutination regularly.

Fitzgerald (28) found that immunization of rabbits with *B. rhinoscleromatis* yielded a potent serum which agglutinated four other strains of the organism equally well. These strains were agglutinated only when capsule-free. Small and Julianelle observed that capsule-bearing strains agglutinated only in high concentrations of immune sera, and the flocculation resulting under these conditions gave a compact disk at the bottom of the tube with a clear supernatant fluid. Capsule-free strains on the other hand agglutinated in serum dilutions as high as 1:2500 and gave a finely granular precipitate which was easily broken up. The encapsulated strains, moreover, adsorbed the specific agglutinins from the immune sera, while capsule-free strains did not.

It becomes evident from this review of the literature that great confusion exists as to the immunological identity of members of the *encapsulatus* group. The impression is that agglutination not only is difficult, but unreliable and not sharply differential. It seemed not unlikely, however, that the application to this group of the principles governing the immunological relationships of pneumococci and their cell constituents might afford a basis for determining similar relations

A BIOLOGICAL CLASSIFICATION OF ENCAPSULATUS PNEUMONIÆ (FRIEDLÄNDER'S BACILLUS).

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In a previous study (1) of the classification of the *encapsulatus* group of organisms certain technical difficulties were encountered which made a serological differentiation of these bacilli confusing and impracticable. The data presented at that time suggested the existence of two types, but these were ill defined and comprised only a small number of all the strains studied. The difficulties attending the immunological classification were ascribed to the failure to obtain potent sera and to the interference of the capsule of the organisms with agglutination. Numerous other investigators had made unsuccessful attempts at a classification, also, and for the most part they have attributed their failures to one or both of these causes.

A review of the literature reveals how contradictory and confusing the data are on the subject. Kraus (2) and Landsteiner (3) were the first to demonstrate agglutination in the *encapsulatus* group. Both investigators procured sera which agglutinated only in low dilutions. Sicard (4), Defalle (5), Clairmont (6), and Porges (7) obtained antisera inconstantly. Immune sera were procured by Klemperer and Scheier (8) and Bertarelli (9) but the sera were not specific and agglutinated closely related species. Specific sera were obtained by Friel (10), Coulter (11), Small and Julianelle (1), and Ball (12).

Howard (13) protected guinea pigs against Friedländer infection by active immunization, but the serum of these animals conferred no passive protection on normal animals. Landsteiner, Sicard, and Clairmont also were unable to demonstrate protective antibodies in the sera of immunized animals. Perkins (14) found that guinea pigs surviving a spontaneous Friedländer epidemic acquired immunity against the organism isolated during the epidemic. In a later paper (15) he states that "animal inoculation, immunization and agglutination show results which are far too variable to admit of them as means for classification."

Limiting their work to the differentiation of the Friedländer and rhinoscleroma organisms, Erben (16), Ballner and Reibmayr (17), and Galli-Valerio (18, 19)

Immunization.—Normal rabbits were bled before immunization was begun and the normal sera were pooled and used for control in the subsequent tests. The animals were immunized by intravenous injections of suspensions of the heat-killed encapsulated organisms. Small doses were injected in the beginning and the amount increased during the course of the injections. Immunization was effected by injecting the organisms on 3 successive days, followed by a rest period of 4 days. Before each course of injections, the titer of the serum of each rabbit was determined; and when the serum showed no further increase in titer, immunization was discontinued. The rabbits were bled 9 or 10 days after the last injection. By this method, an efficient agglutinating serum was obtained in from 4 to 5 weeks.

Agglutination.—Agglutinations were conducted with saline suspensions of living bacilli kept without preservative. The various suspensions were diluted to an equal turbidity before being used for the test. Incubation was carried out for 2 hours at 37°C. in the water bath, and final readings were made after the tubes had been in the ice box overnight. In the more concentrated sera, positive results were usually observable within a few minutes. In agglutinin adsorption tests, heat-killed organisms were used.

Protection.—The protective power of the sera was determined by injecting white mice intraperitoneally with a mixture of 0.2 cc. of the serum and varying dilutions of a broth culture of the organism, both being brought to a volume of 0.5 cc. with sterile broth. The culture used was grown from 4 to 6 hours. The mice were kept under observation for 6 days and those surviving for this period were considered effectively protected.

Precipitin Tests.—In the precipitin tests 0.2 cc. of immune serum diluted to a volume of 0.5 cc. with salt solution was added to an equal volume of increasing dilutions of the soluble specific substance of Friedländer's bacillus. The mixtures were incubated for 2 hours at 37°C. in the water bath. Final readings were made on the following morning after the mixtures had been overnight in the ice box.

Thread Reaction.—In performing these tests, dilutions of the various sera were made in sterile broth. The serum broth tubes were then inoculated with 0.1 cc. of a 4 hour culture of the organisms to be studied and incubation was carried on overnight, for a period of 16 to 18 hours.

Agglutination.

Rabbits were immunized as described to seven of the strains studied. Use was also made of Type II antipneumococcus serum, in view of the recent work of Avery, Heidelberger, and Goebel (38).

The type-specific agglutinations are characteristic and result in a voluminous, compact disk at the bottom of the tube with a clear supernatant fluid. In the lesser dilutions the flocculation occurs immediately and becomes apparent even before incubation.

among the encapsulated bacilli. This newer concept of the bacterial cell (29-34) involves two separable and distinct constituents—the one a polysaccharide, the soluble specific substance, which endows the cell with type specificity; the other a protein substance, which, regardless of type derivation, exhibits immunologically only the common and undifferentiated characteristics of the species. The dissociation of the polysaccharide from the cell deprives the organism of its type-specific antigenic power. In general, antipneumococcus serum contains both the type-specific and the species-specific antibody; the occurrence of the latter depending upon the extent of the dissociation of the antigenic complex, which may take place both *in vitro* and *in vivo*. The presence of the protein antibody in appreciable amounts may therefore mask and sometimes obliterate the type specificity of an organism. Moreover, under unfavorable conditions, these organisms are known to lose the function of elaborating the soluble specific substance—a condition resulting in loss of type specificity, capsule formation, and accompanied by loss of virulence. These degraded strains lose the property of stimulating the type-specific antibody and as antigen provoke only the common protein response (also Stryker (35), Reimann (36), Amoss (37)). In other words, type specificity is demonstrable only when the antisera are dominantly type-specific and the strains to be agglutinated must be not degraded but encapsulated. Stripping an organism of its capsule therefore will minimize the specific serologic reactivity of the organism. It was with this conception, then, that the problem of the classification of the Friedländer bacilli was subjected to further analysis.

EXPERIMENTAL.

Strains of Microorganisms.—The organisms used in this investigation were all members of the *encapsulatus* group as determined by their individual, cultural, staining, and biochemical characteristics. They had been isolated at different times and places within the past 6 years. Strains E and K were isolated from guinea pigs with pneumonia; Strains H1 and H2 were derived from infections in horses; and the remaining strains, from human sources, the majority of them having been associated with lobar pneumonia. Two strains of *Encapsulatus granulomatis*, and two strains of *B. aerogenes* were included in the agglutination tests as a control of specificity. All the strains had in common the production of mucoid, gummy growth, though there were variations in this respect by the individual strains.

The cross-agglutination reactions are summarized in Table I. It becomes evident from the data that anti-Friedländer sera cause a highly specific agglutination which serves to define distinct type relations. Three types are revealed. Of the thirty strains included in

TABLE II.

Agglutinin Adsorption Reaction.

Result of Agglutination with Type A Serum after Adsorption with Strains of Homologous and Heterologous Types.

Strain.	Type.	Serum Type A (F5).						
		Unadsorbed.	Adsorbed by strains of					
			Type A.					Type B.
			F5	F15	Sc	Sm	Bu	E
F3	A	++++	—	—	—	—	—	++++
F5	A	++++	—	—	—	—	—	++++
F8	A	++++	—	—	—	—	—	++++
F9	A	++++	—	—	—	—	—	++++
F15	A	++++	—	—	—	—	—	++++
F18	A	++++	—	—	—	—	—	++++
F19	A	++++	—	—	—	—	—	++++
F20	A	++++	—	—	—	—	—	++++
F21	A	++++	—	—	—	—	—	++++
F23	A	++++	—	—	—	—	—	++++
F25	A	++++	—	—	—	—	—	++++
Sc	A	++++	—	—	—	—	—	++++
Sm	A	++++	—	—	—	—	—	++++
St	A	++++	—	—	—	—	—	++++
Bu	A	++++	—	—	—	—	—	++++
E	B	—	—	—	—	—	—	—

The final dilution of serum in these tests was 1:5.

++++ indicates complete, compact agglutination; — indicates no agglutination.

the survey, twenty-four fall into one or another of these types. For the sake of convenience, these groups will be referred to as Type A, which is composed of fifteen strains; Type B, which is composed of six strains; Type C, which comprises three strains. The remaining six cultures, against four of which no sera were prepared, are placed in a

TABLE I.

Cross-Agglutination Reactions with Strains of Friedländer's Bacillus.

Strain of organism.	Immune sera—final dilution 1:5.								Normal Serum.
	F5	Sc	F2	F6	Pn. II	F10	F11	F12	
F3	++++	++++	—	—	—	—	—	—	—
F5	++++	++++	—	—	—	—	—	—	—
F8	++++	++++	—	—	—	—	—	—	—
F9	++++	++++	—	—	—	—	—	—	—
F15	++++	++++	—	—	—	—	—	—	—
F18	++++	++++	—	—	—	—	—	—	—
F19	++++	++++	—	—	—	—	—	—	—
F20	++++	++++	—	—	—	—	—	—	—
F21	++++	++++	—	—	—	—	—	—	—
F23	++++	++++	—	—	—	—	—	—	—
F25	++++	++++	—	—	—	—	—	—	—
Bu	++++	++++	—	—	—	—	—	—	—
Sc	++++	++++	—	—	—	—	—	—	—
Sm	++++	++++	—	—	—	—	—	—	—
St	++++	++++	—	—	—	—	—	—	—
F2	—	—	++++	++++	++++	—	—	—	—
F6	—	—	++++	++++	++++	—	—	—	—
E	—	—	++++	++++	++++	—	—	—	—
H1	—	—	++++	++++	++++	—	—	—	—
H2	—	—	++++	++++	++++	—	—	—	—
K	—	—	++++	++++	++++	—	—	—	—
F10	—	—	—	—	—	++++	—	—	—
M	—	—	—	—	—	++++	—	—	—
R	—	—	—	—	—	++++	—	—	—
F11	—	—	—	—	—	—	++++	—	—
F12	—	—	—	—	—	—	—	++++	—
F1	—	—	—	—	—	—	—	—	—
F13	—	—	—	—	—	—	—	—	—
F22	—	—	—	—	—	—	—	—	—
F24	—	—	—	—	—	—	—	—	—
G1	—	—	—	—	—	—	—	—	—
G2	—	—	—	—	—	—	—	—	—
A	—	—	—	—	—	—	—	—	—
W	—	—	—	—	—	—	—	—	—

++++ indicates complete, compact agglutination with clear supernatant;
 — indicates no agglutination.

Strains G1 and G2 are strains of *granuloma bacillus*, and A and W, *aerogenes*.

None of these strains were agglutinated by Type I or III antipneumococcus sera. The agglutination titer of these sera was as follows: F5, 1:40; Sc, 1:40; F2, 1:80; F6, 1:40; Pn. II, 1:40; F10, 1:40; F11, 1:160; F12, 1:80.

The B type of the Friedländer bacillus is similar immunologically to Type II Pneumococcus. Avery, Heidelberger, and Goebel have already called attention to this relationship. It will be recalled that they showed that certain strains of Friedländer's bacillus, now designated as Type B, possess specific polysaccharides which, both in chemical and immunological properties, are so similar as to show cross-agglutination and protection, but no reciprocal adsorption of antibodies. It is seen that the six strains of this type react as well with Type II antipneumococcus serum as they do with their homologous sera.

TABLE V.

Protective Action of Anti-Friedländer Serum, Type A, against Strains of Homologous and Heterologous Types.

Amount of culture.	Immune serum Type A.	Strains of Friedländer bacillus.			
		Type A.			Type B.
		F5	F9	Sc	E
cc.	cc.				
10^{-3}	0.2	D. 23 hrs.	D. 48 hrs.	D. 24 hrs.	D. 16 hrs.
10^{-4}	0.2	S.	S.	S.	" 40 "
10^{-5}	0.2	"	"	"	" 40 "
10^{-6}	0.2	"	"	"	" 90 "
10^{-5}	0	D. 15 hrs.	D. 24 hrs.	D. 40 hrs.	D. 16 hrs.
10^{-6}	0	" 20 "	" 45 "	" 45 "	" 44 "
10^{-7}	0	" 40 "	" 46 "	" 46 "	" 40 "

S. indicates that the animal survived; D., that the animal died. The numerals represent the number of hours before death occurred.

Agglutinin Adsorption.

Further evidence of the specificity of the types just mentioned was obtained by agglutinin adsorption tests. The type sera were adsorbed with heat-killed suspensions of both homologous and heterologous type strains. When all the antibodies had been removed for the adsorbing strain, the sera were tested for the presence of agglutinins for the other strains of the homologous type.

The data summarized in Table II show that Serum F5 (Type A)

heterogeneous group, Group X. It is not unlikely that, with an extension of this study so as to include a greater number of strains,

TABLE III.

Agglutinin Adsorption Reaction.

Results of Agglutination with Type B Serum after Adsorption with Strains of Homologous and Heterologous Types.

Strain.	Type.	Serum Type B (F2).				Strain.	Type.	Serum Type B (F6).			
		Unadsorbed.	Adsorbed by strains of					Unadsorbed.	Adsorbed by strains of		
			Type B.		Type A.				Type B.		Type A.
			F2	E	Sc				F2	F6	Sc
F2	B	++++	-	-	++++	F2	B	++++	-	-	++++
F6	B	++++	-	-	++++	F6	B	++++	-	-	++++
E	B	++++	-	-	++++	E	B	++++	-	-	++++
K	B	++++	-	-	++++	K	B	++++	-	-	++++
H1	B	++++	-	-	++++	H1	B	++++	-	-	++++
H2	B	++++	-	-	++++	H2	B	++++	-	-	++++
Sc	A	-	-	-	-	Sc	A	-	-	-	-

TABLE IV.

Agglutinin Adsorption Reaction.

Results of Agglutination with Type C Serum after Adsorption with Strains of Homologous and Heterologous Types.

Strain.	Type.	Serum Type C (F10).				
		Unadsorbed.	Adsorbed by strains of			
			Type C.			Type A.
			F10	M	R	Sc
F10	C	++++	—	—	—	++++
M	C	++++	—	—	—	++++
R	C	++++	—	—	—	++++
Sc	A	—	—	—	—	—

additional types may be demonstrated among the heterogeneous strains of Group X.

Protection Tests.

That Friedländer bacilli are separable into at least three specific types, as demonstrated by the reactions of agglutination and agglutinin adsorption is a fact which gains further demonstration from animal protection tests. It is evident from the data presented in Tables V and VI that the protective action of the type immune sera reveals type specificity in the case of Friedländer bacilli just as sharply as does agglutination. Type A serum (Strain F5) protected mice against homologues of this type to an infective dose of 0.0001 cc. when the cultures themselves killed white mice regularly in a dilution of 0.0000001 cc. within 48 hours. On the other hand it offered no protection against an infection with an organism of a different type. Type B serum (F2) protected against infection by three strains of the homologous type to 0.0001 cc. when the cultures as such were fatal to a dilution of 0.0000001 cc.; yet this serum offered no protection against infection of a different type.

It was not possible to obtain data for the protective power of Type C sera. The three cultures of this type were avirulent for mice, and, despite numerous animal passages, the lethal dose could not be increased. The strains were all capsule-bearing and no "R" colonies could be demonstrated by plating directly from the peritoneal fluid of infected mice.

It is obvious from the results of the protection test that certain anti-Friedländer sera offer definite protection against infection of the organisms and that the protection is type-specific. The protective power of these sera was not as high as had been anticipated however. Avery, Heidelberger, and Goebel obtained a greater degree of protection against Type B infection; but there is no essential discordance between their results and ours, since our animals did not receive as intensive an immunization.

Thread Reaction.

Still further proof of the type specificity of the Friedländer organisms was obtained by means of the thread reaction. This reaction depends upon the character of the growth of an organism in homologous immune serum. Although the mechanism of the reaction may be

was adsorbed with five homologous strains, F5, Sc, F15, Sm, and Bu, and in each case the agglutinins were completely adsorbed. Adsorption with a heterologous strain, E, left untouched the agglutinins in this serum. When Serum F2 (Type B) was adsorbed with Strains F2 and E, homologous strains, agglutinins were removed from the serum not only for the adsorbing strains but for all the strains which were agglutinated by the unadsorbed serum. Strain Sc, a heterologous type, however, exerted no influence on the agglutination of the strains of the type serum. Similarly, adsorption of Serum F6 (also Type B) with

TABLE VI.

Protective Action of Anti-Friedländer Serum Type B, against Strains of Homologous and Heterologous Types.

Amount of culture.	Immune serum Type B.	Strains of Friedländer bacillus.			
		Type B.			Type A.
		F6	K	E	F9
cc.	cc.				
10 ⁻²	0.2	D. 23 hrs.	—	—	—
10 ⁻³	0.2	S.	D. 40 hrs.	D. 40 hrs.	D. 19 hrs.
10 ⁻⁴	0.2	"	S.	S.	" 23 "
10 ⁻⁵	0.2	"	"	"	" 92 "
10 ⁻⁶	0.2	"	"	"	" 40 "
10 ⁻⁶	0	D. 44 hrs.	D. 16 hrs.	D. 16 hrs.	D. 24 hrs.
10 ⁻⁶	0	" 48 "	" 18 "	" 44 "	" 40 "
10 ⁻⁷	0	S.	" 21 "	" 44 "	" 44 "

homologous strains removed all agglutinins for members of this group, whereas, adsorption by Sc, a heterologous strain, again had no effect on the agglutinin titer (Table III). Serum F10 (Type C) was no longer agglutinating for its three homologous strains after adsorption had taken place by Strains F10, M, and R. Strain Sc (Type A), however, adsorbed out none of the agglutinins (Table IV). The results obtained by the method of agglutinin adsorption constitute conclusive evidence that the types disclosed by agglutination with anti-Friedländer sera are real types.

the same as that involved in agglutination, it is usually more delicate than agglutination, and frequently positive results are obtained at a dilution of serum beyond the range of agglutination.

The findings are presented in Table VII. It is seen that a strain gives the thread reaction only when it is grown in immune serum of the homologous type. The reaction extends over the same range of dilutions as agglutination. It was expected that the thread reaction would be detectable in greater dilutions, but the growth of the organism is so profuse as probably to obscure the clumped growth that might occur at the higher dilutions.

Precipitin Reaction.

It has been stated earlier in this paper that a part of the difficulty in demonstrating types by serological reactions in the *encapsulatus* group has been explained on the basis that capsules inhibit agglutination and interfere with agglutinin formation in the body. The methods which were devised to strip the organism of its capsule, at the same time removed from the organism its specificity.

The first workers considered the capsule to be a nucleoprotein (39, 7, 28, 11). The chemical nature of the substance was studied by Rettger (40), who considered it a glycoprotein, possibly a pseudomucin. Toenniessen (41) isolated from a strain of Friedländer's bacillus a polysaccharide which yielded on hydrolysis an osazone, probably of galactose. Kramár (42) isolated a polysaccharide which on inversion yielded galactose. None of these investigators, however, recognized the relationship between the chemical nature and biological specificity of the capsular material.

Recently Mueller, Smith, and Litarczek (43) derived from a Friedländer bacillus a carbohydrate-containing material which reacted to a very high dilution of homologous immune serum. Heidelberger, Goebel, and Avery (44, 38) obtained a nitrogen-free polysaccharide from Strain E (Type B) which reacted specifically with immune serum of the homologous type in dilutions of 1:4 millions. Blake (45) was the first, and as far as we know the only one, to show the appearance of the soluble specific substance of Friedländer's bacillus in the blood and urine during an active infection. Although he could demonstrate no agglutinins in the serum of a patient, he found increasing amounts of the soluble specific substance of Friedländer's bacillus in both blood and urine. This is an interesting example of similarity in Friedländer and *Pneumococcus pneumonia*, since it is possible in both infections (46) to demonstrate the presence of the soluble specific substance of the causative organism in the blood and urine of patients suffering from severe infection.

TABLE VII.

Thread Reaction.

Thread Formation during Growth of Friedländer's Bacillus in Presence of Immune Sera of Homologous and Heterologous Types.

Anti-Friedländer serum.	Strain S ₆ , Type A.						Strain E, Type B.						Strain M, Type C.						Broth control.
	Dilution of serum.						Dilution of serum.						Dilution of serum.						
	1:5	1:10	1:50	1:100	1:500	1:1000	1:5	1:10	1:50	1:100	1:500	1:1000	1:5	1:10	1:50	1:100	1:500	1:1000	
Type A	+++	+++	+	-	-	-	+++	+++	+	+	-	-	-	+++	+++	+	-	-	-
Type B	-	-	-	-	-	-	+++	+++	+	+	-	-	-	+++	+++	+	-	-	-
Type C	-	-	-	-	-	-	+++	+++	-	-	-	-	+++	+++	+	-	-	-	-
Group X	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Normal serum.	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

+++ indicates growth was compact and completely sedimented with clear supernatant; ++ indicates marked, but not complete sedimentation; +, clumping, more easily broken up; ±, partial clumping; -, slight clumping; --, no clumping.

without its capsule is devoid of type specificity. It becomes obvious, therefore, that by removal of the capsule, specificity is lost.

DISCUSSION.

Although numerous attempts have been made by various investigators to classify the Friedländer group of bacilli on the basis of serological reactions, confusion rather than system has resulted. The newer conception of the immunological relationships of the capsular and somatic substances of the bacterial cell has furnished us, in part at least, with an explanation of the earlier failures. The principles involved are those brought out by the immunochemical studies on the type specificity of *Pneumococcus*, which have been carried on in this laboratory. The work has revealed a cellular mechanism in which two distinct and separable substances are concerned in the antigenic and serological behavior of the cell. One of these, the capsular or ectoplasmic layer of the cell, is the soluble specific substance which is now identified as a polysaccharide and is chemically different for each of the fixed types of *Pneumococcus*. The differential specificity of the bacterial types is dependent solely upon the presence of this substance.

That the carbohydrate fraction of the Friedländer bacillus is the substance which determines specificity among organisms of the *encapsulatus* group has been demonstrated in the present study, and in other papers from this laboratory. Removal of the capsule either by hydrolysis or by cultural methods results in a loss of specificity of the organism.

The second constituent of the *Pneumococcus* cell is the somatic protein substance which, regardless of type derivation, exhibits immunologically only the common and undifferentiated characteristics of the species. Immunization by a number of workers with a capsule-free Friedländer bacillus has yielded a serum which reacted with capsule-free strains of any type of these organisms.

Antigenically, the polysaccharide of the bacterial cell is inert. However, in the form in which it exists in the cell, this carbohydrate complex is the dominant and effective antigen yielding on immunization the corresponding type-specific antibody. It is now recognized that cell dissolution, whether occurring spontaneously in the body or arti-

Since the relation of capsular material and specificity has been recognized it seemed desirable to determine the action of immune sera of the different types on the isolated specific substance of Friedländer bacilli. Nitrogen-free polysaccharides of Strain Sc (Type A) and Strain E (Type B) were obtained¹ and cross-precipitin reactions were carried out in the usual manner. The amount of immune serum was kept constant at 0.2 cc. while the polysaccharide was used in increasing dilutions.

TABLE VIII.

Precipitation of the Soluble Specific Substance of Friedländer Bacilli by Immune Sera of Homologous and Heterologous Types.

Anti-Friedländer serum.		Type A soluble specific substance.								Type B soluble specific substance.								Salt control.
		20*	50	100	250	500	1000	2000	4000	20	50	100	250	500	1000	2000	4000	
Type A	F5	++	++	+	±	-	-	-	-	-	-	-	-	-	-	-	-	-
	Sc	++	++	++	+	±	-	-	-	-	-	-	-	-	-	-	-	-
Type B	F2	-	-	-	-	-	-	-	-	+	++	++	++	++	±	-	-	-
	F6	-	-	-	-	-	-	-	-	+	++	++	+	++	±	-	-	-
	E	-	-	-	-	-	-	-	-	+++	+++	+++	+	++	±	-	-	-
Type C	F10	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Group X	F11	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	F12	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Normal serum.		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

++++ indicates heavy compact, disk precipitate; +++, marked disk precipitate; ++, thin, film-like scale; +, ground glass turbidity; ±, slight turbidity; -, no reaction:

* These figures represent dilutions in thousands.

The results of the precipitation tests are presented in Table VIII, and they show that the polysaccharide of each type reacts only with immune sera of the corresponding type and that precipitation occurs at a high dilution of the soluble specific substance. Further evidence to be reported in a subsequent publication will show that the organism

¹ The polysaccharides were obtained through the courtesy of Dr. Heidelberger and Dr. Goebel.

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ficially *in vitro*, is accompanied by more or less antigenic dissociation. The result of this process is reflected in the immune serum by the increasing amount of non-specific antibody with diminution or complete loss of type-specific response.

In previous studies it was observed that prolonged immunization induced a serum of high titer for the Friedländer organism, but that the serum agglutinated as well a number of organisms which were unable to remove agglutinins of the specific type by adsorption of the serum. Other workers also produced high titered sera at the expense of specificity.

The lack of recognition of two distinct antibodies in a single immune serum, each specifically reactive with only one of the cell constituents, and conversely the failure to distinguish the type-specific from the species-specific antigen of the cell, explain some of the difficulties encountered in the earlier efforts at classification of the *encapsulatus* group.

In a later paper the immunological relationships of these two constituents the protein and carbohydrate, and of encapsulated and capsule-free strains of the Friedländer bacillus, will be described.

SUMMARY.

A biological classification has been made of thirty strains of Friedländer's bacillus. This study reveals that there exist among these strains three sharply defined and specific types and one heterogeneous group. The three types are Type A, fifteen strains; Type B, six strains; Type C, three strains; and Group X, six strains. The agglutination, agglutinin adsorption, protection, thread, and precipitin reactions have been employed in the working out of this classification, and the types have been proved highly specific by means of each serological test.

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importance of the spleen in sensitization, only short notes by Siegmund (11) and Neufeld (12) are found in the literature on the relation of the cells of the reticulo-endothelial system to the occurrence of anaphylaxis.¹ While Siegmund states that blocking of the reticulo-endothelial system led to the prevention of anaphylactic shock without, however, mentioning whether the blocking injections were given before or after sensitization, Neufeld reports that he has been unable to prevent sensitization in mice and guinea pigs by blocking and splenectomy carried out before the sensitizing injection.

In view of the meager and variable results obtained on the effect of blockade before sensitization in the occurrence of anaphylaxis, it seemed desirable to enter into a detailed study of the subject. Moreover, such work, if it were to include a quantitative titration of the anaphylactic power of the serum and a determination of the precipitin titer, would be of interest in throwing new light on the theory of the identity of precipitins and the anaphylactic antibody as advanced by Doerr (16). The study was therefore arranged to include the following points: (1) the effect upon the development of active anaphylaxis of India ink injected intravenously into guinea pigs prior to the sensitizing injection; (2) a determination of the precipitin titer and titration of the anaphylactic power of the serums of rabbits, injected intravenously with India ink prior to the sensitizing injections; (3) the effect upon the development of passive anaphylaxis in guinea pigs of intravenous injections of India ink given prior to the sensitizing injection. At the same time it was thought of interest not only to study the influence of massive blocking doses, but also of smaller doses of India ink, on the occurrence of these phenomena. Animals which had received massive doses of ink intravenously are frequently referred to later as "blocked," merely for the sake of brevity and not with the intention of making a definite statement as to the mode of action of the ink. .

¹ Since this study was completed, Fujioka (13), Isaacs (14), and Simitch (15) have reported experimental work on the same subject. Isaacs has seen no shock-preventing action of trypan blue given either before or after sensitization in actively sensitized guinea pigs. Likewise Simitch observed that after blocking with such substances as iron oxide and India ink before sensitization, the occurrence of anaphylaxis was not interfered with. Fujioka, on the other hand, found that after experimental disturbance of functions of the reticulo-endothelial cells by the administration of lanolin emulsion, both active and passive anaphylaxis could be prevented.

THE RÔLE OF THE RETICULO-ENDOTHELIAL SYSTEM IN IMMUNITY.

III. THE PRODUCTION OF ACTIVE AND PASSIVE ANAPHYLAXIS IN THE BLOCKED ANIMAL.

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INTRODUCTION.

With the increase of experimental work on the significance of the reticulo-endothelial tissue, especially that found in the liver and spleen, for the allergic reaction of the immune animal, the opinion seems to be more widely accepted that there is an intimate relationship between antibody production and the reticular elements of the organs mentioned.

Successful experiments to inhibit or suppress the formation of immune bodies (agglutinins, precipitins, hemolytic amboceptors) by blocking injections of India ink and other substances of colloidal dispersion, were reviewed thoroughly in a previous paper (1) in which the authors were able to show that no diphtheria antitoxin appeared in the blood during a period of 3 weeks in guinea pigs injected intravenously with massive doses of India ink before immunization with diphtheria toxin-antitoxin mixture. In a later paper (2), it was demonstrated that the complement titer of the serums of guinea pigs after intravenous injections of ink showed a marked transitory drop. Simultaneously, the regenerative power of the reticulo-endothelial cells of the liver and spleen of guinea pigs which had received one blocking injection of India ink, was measured by means of reduction tests. In these experiments it was found that, physiologically, the integrity of these tissues was restored in from 24 to 48 hours.

While the influence of intravenous injections of colloidal substances in the anaphylactic animal before reinjection of the antigen has recently been studied by a number of authors (3-8), it remains to be investigated whether massive doses of India ink, given before the sensitizing injection, will prevent or modify the sensitization of an animal. Aside from the earlier experiments of Mautner (9), more recently confirmed and amplified by Luzzato (10), which demonstrated the

EXPERIMENTAL WORK.

A. Experiments on the Effect of Blockade before Active Sensitization on the Occurrence of Anaphylactic Shock in Guinea Pigs.

First Series.—Six guinea pigs of from 230 to 280 gm. in weight were given two intravenous injections of 1.5 cc. India ink,² diluted with physiological salt solution 1:5; simultaneously, six guinea pigs received one intravenous injection of 1 cc. India ink, diluted 1:20. 3 days after the last ink injection, all twelve animals, together with six normal controls, were sensitized by one subcutaneous injection of 0.05 cc. of normal horse serum (1 cc. of a dilution 1:20). The intravenous reinjection was carried out 3 weeks later. Great care was taken to determine as accurately as possible the smallest dose of antigen which would kill a normal sensitized guinea pig with certainty, and this dose was later used for the reinjection of the ink-treated animals. This was necessary in order to facilitate the detection of very small changes in the degree of sensitization. The results obtained in this series are given in Table I.

It appears from this table that of the six blocked animals one survived, four died in a somewhat protracted shock, and only one died in 3 minutes with a dose (0.2 cc.) that, in from 3 to 4 minutes, produced typical shock in, and killed, the four non-blocked controls that received it. On the other hand, four out of six animals that had received the smaller dose of ink showed the same sensitiveness as the controls, when tested with the 0.2 cc. of normal horse serum, but the remaining two animals when injected with a dose sublethal for controls (0.175 cc.) died of acute shock in 4 and 10 minutes respectively. The results seem to indicate that under the conditions of the experiment massive doses of India ink injected intravenously before subcutaneous sensitization may effect a slight reduction in sensitiveness to the reinjection of the antigen 3 weeks later.

It was thought to be of interest to investigate whether the serum of the blocked guinea pigs which had exhibited a slightly decreased sensitiveness as compared with normal controls, contained enough anaphylactic antibody to make possible a passive transfer.

For this purpose, 4 cc. of blood were obtained by cardiac puncture from two blocked animals and from two controls 2 days before the reinjection in the ex-

² The technique of the intravenous injection was described in detail in the first paper. The India ink used was Higgins' insoluble India ink, which has been employed throughout these studies.

TABLE I.
Series 1.
Production of Active Anaphylaxis in the Blocked Guinea Pig.

Guinea pig No.	Blocking injections of ink.*			Sensitizing injections of normal horse serum No. 140 on June 15.†	Reinjections of normal horse serum No. 140 on July 6.*	Results.
	Date.	Dilution.	Dose.	Dose.	Dose.	
1			cc.	cc.	cc.	Moderate symptoms. Survived.
2				0.05	0.175	" " "
3				0.05	0.2	Died in 3 min.
4				0.05	0.2	" " 5 "
5				0.05	0.2	" " 3 "
6				0.05	0.2	" " 8 "
7	June 7	1:5	1.5	0.05	0.2	" " 3 "
	" 12	1:5	1.5			
8	" 7	1:5	1.5	0.05	0.2	Moderate symptoms. Died in 40 min.
	" 12	1:5	1.5			
9	" 7	1:5	1.5	0.05	0.2	Severe symptoms. Died in 2 hrs.
	" 12	1:5	1.5			
10	" 7	1:5	1.5	0.05	0.2	Slight symptoms. Died in 22 hrs.
	" 12	1:5	1.5			
11	" 7	1:5	1.5	0.05	0.2	Severe symptoms. Died in 20 min.
	" 12	1:5	1.5			
12	" 7	1:5	1.5	0.05	0.2	Moderate symptoms. Survived.
	" 12	1:5	1.5			
13	" 12	1:20	1	0.05	0.2	Died in 5 min.
14	" 12	1:20	1	0.05	0.2	" " 6 "
15	" 12	1:20	1	0.05	0.2	" " 3 "
16	" 12	1:20	1	0.05	0.2	" " 4 "
17	" 12	1:20	1	0.05	0.175	" " 4 "
18	" 12	1:20	1	0.05	0.175	" " 10 "

The symptoms consisted of the classical syndrome, but varied in intensity. The autopsy findings were typical in each case of acute shock; lungs were maximally inflated; congestion of abdominal organs was present; heart's blood was found not clotted.

* All blocking injections and reinjections were intravenous.

† All sensitizing injections were subcutaneous.

In this series, six guinea pigs, of from 250 to 280 gm. in weight, were given two intravenous injections of 1.5 cc. of a 1:5 dilution of ink at 6 day intervals and were

TABLE III.

Series 2.

Production of Active Anaphylaxis in the Blocked Guinea Pig.

Guinea pig No.	Blocking injections of ink 1:5 dilution.*		Sensitizing injections of normal horse serum No. 140 on July 20.*	Reinjections of normal horse serum No. 140 on Aug. 10.*	Results.
	Date.	Dose.	Dose.	Dose.	
24		cc.	0.01	0.2	Slight symptoms. Survived.
25			0.01	0.5	Moderate symptoms. Survived.
26			0.01	0.5	" " "
27			0.01	0.75	Died in 3 min.
28			0.01	0.75	" " 10 "
29			0.01	0.75	" " 6 "
30	July 14	1.5	0.01	0.75	" " 3 "
	" 20	1.5			
31	" 14	1.5	0.01	0.75	" " 3 "
	" 20	1.5			
32	" 14	1.5	0.01	0.75	" " 4 "
	" 20	1.5			
33	" 14	1.5	0.01	0.75	" " 3 "
	" 20	1.5			
34	" 14	1.5	0.01	0.75	" " 3 "
	" 20	1.5			
35	" 14	1.5	0.01	0.5	Moderate symptoms. Survived.
	" 20	1.5			

Autopsy findings in each case were typical.

* All injections were intravenous.

periment described above was carried out. 2 cc. of serum from each animal were injected intraperitoneally into each of four normal guinea pigs. These guinea pigs were reinjected intravenously 24 hours later with 1 cc. of normal horse serum. Simultaneously, one normal guinea pig was injected intravenously with the same amount to control the toxicity of the horse serum. Table II gives the results obtained.

This table shows that both blocked guinea pigs had developed, as had the controls, enough circulating anaphylactic antibody to make possible a passive transfer. The small number of animals in this case, however, makes the test only qualitative in nature and does not permit

TABLE II.

Passive Anaphylaxis in Normal Guinea Pigs with Serum from Sensitized Blocked and Normal Guinea Pigs.

Guinea pig No.	Sensitizing injection on July 5.*		Reinjections of normal horse serum No. 140 on July 6.†	Results.
	Serum from Guinea pig No.	Dose.	Dose.	
19	5	2	1	Died in 3 min.
20	3	2	1	" " 5 "
21	9	2	1	" " 3 "
22	10	2	1	" " 10 "
23			1	No symptoms.

Autopsy findings in each case were typical.

* All sensitizing injections were intraperitoneal.

† All reinjections were intravenous.

the quantitative comparison of the anaphylactic power of the serums of blocked animals with those of controls.

Second Series.—It was next thought to be of interest to investigate the effect on the occurrence of anaphylactic shock in guinea pigs of intravenous injections of India ink preceding sensitization, which was carried out intravenously. In view of the rapidity with which the reticulo-endothelial system recovers after blockade, it seemed also desirable to reduce the interval between blocking and sensitizing injections to the shortest possible time.

TABLE IV.

Series 3.

Production of Active Anaphylaxis in the Blocked Guinea Pig.

Guinea pig No.	Blocking injections of ink 1:5 dilution.*		Sensitizing injections of normal horse serum on Oct. 27.†	Reinjections of normal horse serum.*		Results.
	Date.	Dose.		Date.	Dose.	
36		cc.	0.002	Nov. 12	0.15	Died in 3 min.
37			0.002	" 12	0.1	" " 3 "
38			0.002	" 12	0.1	" " 4 "
39			0.002	" 12	0.05	Moderate symptoms. Survived.
40			0.002	" 24	0.1	Severe " Died in 20 hrs.
41			0.002	" 24	0.1	Died in 10 min.
42	Oct. 23	1.5	0.002	" 12	0.1	Moderate symptoms. Survived.
	" 26	1.5				
43	" 23	1.5	0.002	" 12	0.1	" " "
	" 26	1.5				
44	" 23	1.5	0.002	" 12	0.1	Died in 10 min.
	" 26	1.5				
45	" 23	1.5	0.002	" 12	0.15	" " 4 "
	" 26	1.5				
46	" 23	1.5	0.002	" 24	0.1	Severe symptoms. Survived.
	" 26	1.5				
47	" 23	1.5	0.002	" 24	0.1	Died in 8 min.
	" 26	1.5				

Autopsy findings were typical in each case, of acute shock.

* All blocking injections and reinjections were intravenous.

† All sensitizing injections were subcutaneous.

sensitized 15 minutes after the last blocking injection by intravenous injection of 0.01 cc. of normal horse serum (1 cc. 1:100). At the same time, six normal controls were sensitized in the same manner. 3 weeks later the blocked animals were reinjected intravenously with the minimum dose of normal horse serum fatal to controls. The results obtained in this series are shown in Table III.

This table fails to disclose any reduction of sensitiveness in the blocked animals, as compared with the normal controls, to the reinjection of the antigen. With the dose of 0.75 cc., invariably fatal to the controls, all blocked animals also died typically, while the dose of 0.5 cc. was followed by the survival of two control animals and one blocked animal. There seems to be a fundamental difference between subcutaneously and intravenously sensitized guinea pigs as regards their susceptibility to anaphylactic shock following blocking injections of India ink given before sensitization.

Third Series.—Since the effect of blocking before sensitization apparently varies, it seemed desirable to run another series with a view to confirming the results obtained in the first series with subcutaneous sensitization. The extreme delicacy of the anaphylactic reaction as an indicator of antibody production, as manifested by the disproportion between the size of the sensitizing dose and the degree of change produced, subjects experiments on the inhibition of antibody production to a test of maximum severity. It was thought that by decreasing the antigenic dose to the smallest stimulus compatible with a regularly occurring sensitization and by testing for anaphylaxis before the condition had reached its maximum development, it should be possible to demonstrate better an inhibiting influence of the blockade on the formation of the anaphylactic antibody.

In this third series, six guinea pigs of from 250 to 280 gm. in weight, were given two intravenous injections of ink, as already described, and were sensitized 1 day after the last ink injection by a subcutaneous injection of 0.002 cc. of normal horse serum (1 cc. 1:500). Simultaneously, six control animals were sensitized with the same dose. Four blocked and four control animals were reinjected intravenously with the definitely fatal dose after an interval of 16 days, and the remaining four animals after 28 days. The results are shown in Table IV.

It appears from Table IV that in the first group, reinjected after 16 days had elapsed since the sensitization, two blocked animals survived and one died after the injection of a single lethal dose (0.1 cc.) which

TABLE V.

Determination of Precipitin Titer of Rabbit Antisera.

Rabbit No.	Trial bleeding.		Dilutions.										
	Date.	Time since last antigen injection.	1:5	1:10	1:20	1:40	1:80	1:100	1:160	1:200	1:320	1:640	1:1000
		<i>days</i>											
1*	July 28		±	—	—								
	Aug. 14	2		+	+	—	—	—	—	—			
	" 16	4		+	+	—	—	—	—	—			
	" 18	6		+	+	+	—	—	—	—	—	—	—
2*	July 28		+	—	—								
	Aug. 14	2		+	+	—	—	—	—	—			
	" 16	4		+	+	+	—	—	—	—			
	" 18	6		+	+	+	+	+	+	—	—	—	—
3†	July 28		±	—	—								
	Aug. 14	2		+	+	+	+	+	+	—			
	" 16	4		+	+	+	+	+	+	+	+		
	" 18	6		+	+	+	+	+	+	+	+	+	—
4†	July 28		±	—	—								
	Aug. 14	2		+	+	+	+	—	—	—			
	" 16	4		+	+	+	+	+	+	—			
	" 18	6		+	+	+	+	+	+	+	—	—	—
5†	July 28		±	—	—								
	Aug. 14	2		+	+	+	+	—	—	—			
	" 16	4		+	+	+	+	+	+	—			
	" 18	6		+	+	+	+	+	+	+	+	—	—
6†	July 28		—	—	—								
	Aug. 14	2		+	+	+	—	—	—	—			
	" 16	4		+	+	+	+	—	—	—			
	" 18	6		+	+	+	+	+	—	—	—	—	—

* Received large doses of ink.

† Received small doses of ink.

‡ Received salt solution.

Technique:

Rabbit serum dilutions 0.5 cc.

Normal horse serum undiluted (constant) 0.5 cc.

Incubation 1 hour at 37°C.

killed two controls in acute shock, while a slightly larger dose (0.15 cc.) produced fatal shock in one blocked animal.

In the second group, one out of two animals survived the injection of a single lethal dose (0.1 cc.) which killed two controls.

B. Determination of the Precipitin Titer and of the Anaphylactic Power of the Serum of Actively Sensitized, Blocked Rabbits.

In the work reported in the first part of this paper it was shown that guinea pigs blocked before active sensitization may exhibit occasionally a slightly lower sensitiveness to the reinjection of the antigen. By means of a rough qualitative test, it was demonstrable that the blocked animals, in spite of their inherent slighter sensitiveness, had accumulated a sufficiently high concentration of circulating antibodies to make possible a passive transfer to normal guinea pigs, resulting in acute death following the injection of the antigen. In order to provide a more concrete explanation of the reduced sensitiveness observed in blocked guinea pigs, it was necessary to carry out a quantitative titration by passive transfer, sufficiently accurate to detect any difference in the titer of anaphylactic antibodies in blocked and in normal animals. Simultaneously, it seemed of interest to study the precipitin titer in such serums in comparison with their anaphylactic power. As these experiments called for greater amounts of serum, rabbits were used for the production of the antiserum and guinea pigs for the passive transfer.

Two rabbits (Nos. 1 and 2) were each given three intravenous ink injections of 5 cc. of a 1:4 dilution at 2 day intervals, and then four intravenous injections of normal horse serum (1 cc., 2 cc., 3 cc., 3 cc.) at 3 day intervals, each time preceded by another ink injection of the same dose. Two rabbits (Nos. 3 and 4) were treated on the same schedule with ink injections of 5 cc. of a 1:50 dilution and sensitized in the same manner. Two control rabbits (Nos. 5 and 6) were given 5 cc. of salt solution intravenously, whenever the other four animals received ink injections, and were sensitized with the same doses. All the animals in each case weighed from 1800 to 2200 gm. Blood was taken before treatment, 2 and 4 days after the last injection, and, on the 6th day, all the animals were bled to death. The precipitin titer of the different serums was not determined by the Uhlenhuth method, but by diluting the antiserum and keeping the antigen constant. The results obtained are shown in Table V.

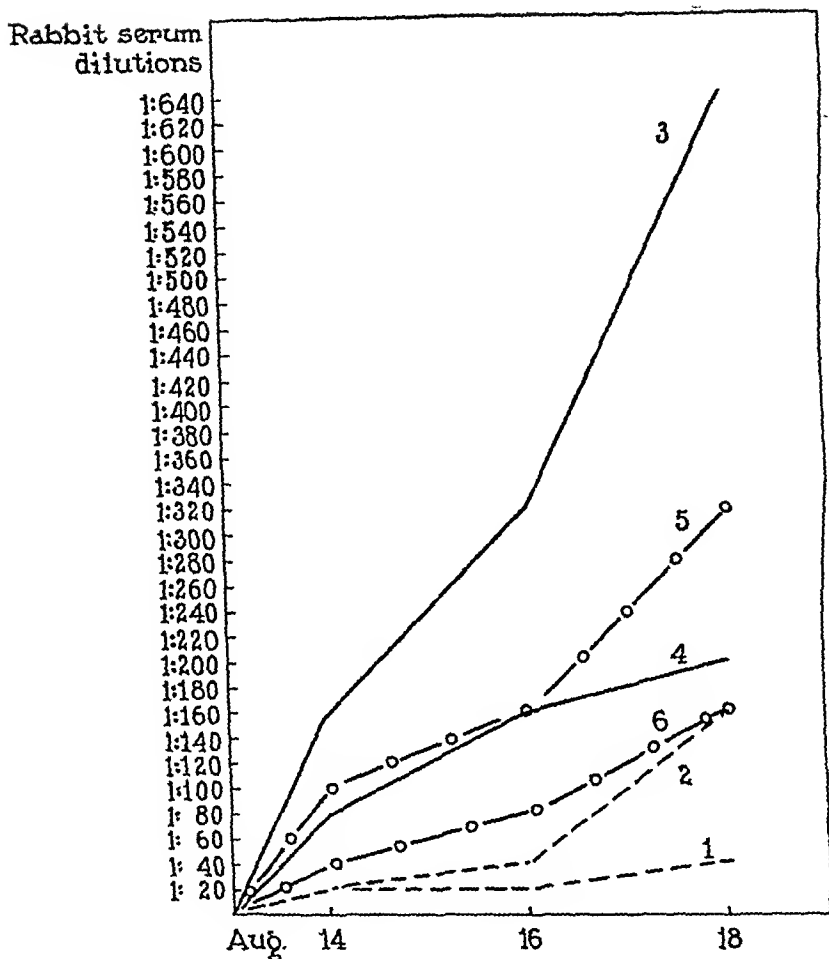
It appears from this table and also from the accompanying graph that the serum from the two blocked animals had the lowest precipitin titer, though eventually one serum reached the same height as one control serum. On the other hand, the serum of one animal which had received the smaller ink doses showed throughout the highest titer of precipitins, while the serum from the other animal treated in the same way had about the same titer as the other control serum.

All six serums (final bleedings) were tested for their anaphylactic power in guinea pigs according to the method outlined by Doerr and Russ (17).

1 cc. of each serum was injected intraperitoneally into each of four guinea pigs, making a total of twenty-four guinea pigs, and 24 hours later all the guinea pigs were injected intravenously with graduated amounts of normal horse serum, four different amounts being used for each particular serum. The results of this test are shown in Table VI.

It appears from this table that the serum from one blocked animal (No. 1) had a slightly lower anaphylactic titer than that of two normal control animals, a dose of 0.05 cc. of normal horse serum being followed by death after only 2 hours, and the doses of 0.02 and 0.01 cc. being tolerated without any symptoms. The anaphylactic titer of the serum of the other blocked animal did not differ appreciably from that of the controls. The anaphylactic titer of the serum of two animals treated with the smaller doses of ink was not different from the titer of the control animals. Comparison with the precipitin titer shows that in one instance (blocked rabbit No. 1 and controls Nos. 5 and 6) a lower precipitin titer ran parallel with a slightly lower anaphylactic titer, while in another instance of a higher precipitin titer (rabbit No. 3 and two controls) no such parallelism was observed.

The results obtained in this experiment were essentially negative. They by no means furnish proof that the production of circulating anaphylactic antibody in the blocked sensitized rabbit is lower than that of the normal sensitized rabbit. It seems justifiable, however, to conclude that the formation of precipitins in blocked sensitized rabbits is temporarily inhibited as compared with normal sensitized control animals. As regards the effect of smaller doses of ink, it would seem that in one case they caused a stimulation in the production of



TEXT-FIG. 1.

— Animals injected with small doses of ink.

--- Animals injected with large doses of ink.

—○—○— Control animals injected with physiological salt solution.

Comparison of the precipitin titer of antisera in rabbits injected with small and large doses of India ink.

Technique:

Titer determined after 1 hour's incubation at 37°C.

Rabbit serum dilutions: 0.5 cc.

Normal horse serum undiluted: 0.5 cc. (constant).

precipitins, while the anaphylactic power of the serum of rabbits treated with these smaller doses was similar to that of the controls.

C. Experiments on the Effect of Blockade before Passive Sensitization on the Occurrence of Anaphylactic Shock in Guinea Pigs.

While the diminution of sensitiveness observed in blocked, actively sensitized guinea pigs might be considered analogous to the interference of blockade in processes of active immunization, no such

TABLE VII.

The Production of Passive Anaphylaxis in the Blocked Guinea Pig.

Guinea pig No.	Blocking injections of ink 1:5 dilution on Aug. 27.*	Sensitizing injection of rabbit serum No. 5 on Aug. 27.†	Reinjections of normal horse serum No. 140 on Aug. 28.*	Results.
	Dose.	Dose.	Dose.	
	cc.	cc.	cc.	
72	1.5	1	0.05	Died in 4 min.
73	1.5	1	0.05	" " 6 "
74	1.5	1	0.02	Moderate symptoms. Survived.
75	1.5	1	0.02	Severe " "
76		1	0.05	Died in 4 min.
77		1	0.05	" " 5 "
78		1	0.02	Severe symptoms. Survived.
79		1	0.02	Slight " "

Autopsy findings in each case were typical.

* Blocking injections and reinjections were intravenous.

† Sensitizing injections were intraperitoneal.

mechanism could be thought of in passive anaphylaxis. Blocking experiments before passive sensitization were done with a view to obtaining data concerning the extent to which cellular changes of the organs of the blocked animals, effected by the absorption of ink, were involved in the reduction of sensitiveness.

Four guinea pigs of from 230 to 280 gm. in weight were given one intravenous injection of 1.5 cc. ink, diluted 1:5, and were passively sensitized 1 day later, together with four normal controls, by intraperitoneal injection of 1 cc. of rabbit antiserum No. 5. All eight guinea pigs were reinjected 24 hours later, four with a

TABLE VI.

Titration of Anaphylactic Power of Rabbit Antiserums by Passive Transfer to Guinea Pigs.

Guinea pig No.	Sensitizing injection.*			Reinjections of normal horse serum No. 140.†		Results.
	Serum from Rabbit No.	Date.	Dose.	Date.	Dose.	
48	1‡	Aug. 21	1	Aug. 22	0.1	Died in 4 min.
49	1	" 21	1	" 22	0.05	Very severe symptoms. Died in 2 hrs.
50	1	" 21	1	" 22	0.02	No symptoms. Survived.
51	1	" 21	1	" 22	0.01	" " "
52	2‡	" 21	1	" 22	0.1	Died in 4 min.
53	2	" 21	1	" 22	0.05	" " 10 "
54	2	" 21	1	" 22	0.02	No symptoms. Survived.
55	2	" 21	1	" 22	0.01	Slight symptoms. Survived.
56	3§	" 21	1	" 22	0.1	Died in 4 min.
57	3	" 21	1	" 22	0.05	" " 4 "
58	3	" 21	1	" 22	0.02	Moderate symptoms. Survived.
59	3	" 21	1	" 22	0.01	No symptoms. Survived.
60	4§	" 22	1	" 23	0.1	Died in 8 min.
61	4	" 22	1	" 23	0.05	" " 10 "
62	4	" 22	1	" 23	0.02	Slight symptoms. Survived.
63	4	" 22	1	" 23	0.01	No symptoms. Survived.
64	5	" 22	1	" 23	0.1	Died in 3 min.
65	5	" 22	1	" 23	0.05	" " 4 "
66	5	" 22	1	" 23	0.02	Severe symptoms. Survived.
67	5	" 22	1	" 23	0.01	Moderate symptoms. Survived.
68	6	" 22	1	" 23	0.1	Died in 3 min.
69	6	" 22	1	" 23	0.05	" " 4 "
70	6	" 22	1	" 23	0.02	Slight symptoms. Survived.
71	6	" 22	1	" 23	0.01	No symptoms. Survived.

Autopsy findings in each case were typical.

* All sensitizing injections were intraperitoneal.

† All reinjections were intravenous.

‡ Received large doses of ink.

§ Received small doses of ink.

|| Received salt solution.

of anaphylactic antibody as that of the controls. The fact that blocking before passive sensitization did not interfere with the occurrence of the anaphylactic shock in any way seems to indicate that, if the animal plays no active part in the production of the anaphylactic antibody, the mere presence of ink, under the conditions of our experiments, does not affect the animal's susceptibility to the anaphylactic reaction as such. Finally, it is of interest to note that there was no complete parallelism between the precipitin titer and the anaphylactic power of the serum of several ink-treated sensitized rabbits. While the lower precipitin titer in a blocked animal in one case corresponded with a slightly lower anaphylactic power, this was not true with the higher precipitin titer and the anaphylactic power of the serum of one rabbit which had received the smaller doses of ink.

SUMMARY AND CONCLUSIONS.

1. Guinea pigs injected intravenously with massive doses of India ink before active sensitization exhibited occasionally a more or less marked, decreased sensitiveness to the reinjection of the antigen.

2. The serum of rabbits which had received massive doses of India ink before the sensitizing injections, showed approximately the same titer of anaphylactic antibodies as that of sensitized normal control animals, as demonstrated by the degree of passive sensitization induced in guinea pigs.

3. The precipitin titer of sensitized rabbits blocked with massive doses of India ink was somewhat lower than that of sensitized normal controls. In one instance, the intravenous injection of smaller doses of India ink was followed by a higher precipitin titer. No uniform relation was found between the height of the precipitin titer and the anaphylactic power of the antiserums.

4. One blocking injection of India ink given to guinea pigs before passive sensitization did not interfere with the occurrence of anaphylactic shock nor alter its nature.

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lethal and four with a sublethal dose of normal horse serum. The results obtained are given in Table VII.

No difference in sensitiveness between the two groups was observed. It would seem justifiable to conclude from this experiment that the occurrence of anaphylactic shock in passively sensitized guinea pigs is not interfered with by giving one blocking injection of India ink before this sensitization.

DISCUSSION.

The observations here recorded agree in principle with the experience of many other authors, that any active immunization process in blocked animals is inhibited in direct proportion to the extent of elimination of the reticulo-endothelial system. This is especially true of the formation of precipitins in the blocked animal as recently reported by Gay and Clark (18). The quick regeneration of the blocked system obviously makes it impossible to obtain more than relative results with the present methods of blockade. It is doubtful whether any procedure can be developed which would accomplish more without endangering vital physiological functions of life, except possibly a combination of blocking with splenectomy.

We feel inclined, in view of the positive results we have obtained before, to ascribe to the reticulo-endothelial system singular importance in the production of antibodies. This opinion is considerably strengthened by the observations made during the course of this study. The precipitin titer of the serum of blocked, actively sensitized rabbits was definitely lower than that of normal sensitized controls, while smaller doses of ink in one case were followed by a higher precipitin titer than that seen in controls. Our experiments demonstrate further that the anaphylactic shock in the majority of guinea pigs which received blocking injections of ink before active sensitization, assumed a definitely milder and more protracted form and that occasionally a blocked, actively sensitized animal will survive even a dose of antigen fatal to the controls. Our experiments do not intimate a convincing explanation for this reduced sensitiveness since a titration by means of passive anaphylaxis in guinea pigs showed the blood serum of blocked, sensitized rabbits to contain approximately the same amount

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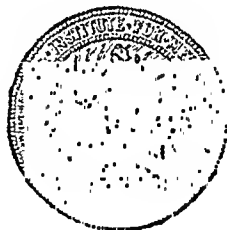
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sections showed that in each the main line of fibroblastic growth was in the direction of the applied tension. Repetitions of the experiment gave the same result, and Figs. 1 and 2 show the difference in direction of growth in Rabbit 10, in which excision of the tissue was made 6 days after the primary incision.

In order to test further the effect of tension, a knife point incision of less than 5 mm. in length was made in the skin of the back and four pair of tension sutures arranged about the cut so as to give a simultaneous centrifugal pull perpendicular to the line of incision, parallel to the line, and along the 45° diagonals. The wound which had been stretched into a small oval ulcer was excised at the end of 7 days and sectioned parallel to the surface. Fig. 3 is a photograph taken from as near the centre as possible of this wound and it shows a well marked radial tendency of growth on the part of the fibroblasts.

These experiments seem to indicate that in connective tissue growing under mechanical tension the spindle-shaped fibroblasts will arrange themselves with their long axis parallel to the line of pull. That this is not simply an elongation of the protoplasm of the cell in a certain direction, but is the expression of a definite polarity in the cell is indicated by a study of the mitotic figures occurring in the sections. In the dividing cells found in the tissues, the figures lie in such manner that a line connecting the poles of the achromatic spindle is in the line of tension, and thus the equatorial plane and the plane of cell division are perpendicular. The two daughter cells thus separate in the line of tension.

In view of the behavior of fibroblasts in granulation tissue, it seemed important to determine whether or not a shift in tension would cause fibroblasts to shift their polarity or position after it was once established. As is well known, in granulation tissue the new fibroblasts at first grow out parallel to the capillary sprouts, and thus, in the surface or youngest part of the granulation, lie perpendicular to the free surface. Deeper down in the granulation they lie in an oblique position and at the base in the older part of the tissue they lie perpendicular to the vessels and parallel to the surface, a position found in the ultimate scar tissue. To test the effect of change in tension, traction was applied perpendicular to the line of a skin incision for 2 days; these sutures were then cut and traction made by a second pair of sutures in the line of the incision and at right angles to the first tension. After 4 days, the tissue was excised and sectioned. Fibroblasts in the scar line were all arranged parallel to the line of incision

THE EFFECT OF MECHANICAL TENSION UPON THE POLARITY OF GROWING FIBROBLASTS.

By C. H. BUNTING, M.D., AND CHARLES C. EADES, M.D.

(From the Pathological Laboratory of the University of Wisconsin, Madison.)

PLATE 4.

(Received for publication, April 23, 1926.)

In the healing of a sterile surgical skin incision the newly formed fibroblasts show a definite polarity which leads them to grow across the wound parallel to the surface and to all practical purposes parallel to each other. The cause of this polarity is not obvious in sections of such growing tissue. However, other pathological new growths of connective tissue give a hint that mechanical tension plays a part in the process. Thus in an organizing pericardial exudate, fibroblasts grow out parallel to the newly formed capillaries and perpendicular to the surface, and eventually come to lie parallel to the surface unless an adhesion is formed between the two pericardial surfaces, when they maintain their original position parallel to the vessels. The experiments here reported are an attempt to establish the effect of mechanical tension upon the polarity of growing fibroblasts.

For the purposes of the experiments incisions were made into the thick corium of the back of anesthetized rabbits, with aseptic precautions. In order to secure variation in the direction of tension upon the healing wound, a wire fly screen saddle was fitted close to the back, permitting deep sutures, placed at selected points, to be brought through the screen at any desired point and tied above so as to maintain pull in the direction wished. At varying intervals such wounds were excised, fixed in Zenker's fluid, and cut in serial sections parallel to the surface. This procedure was adopted after it was found that strips of adhesive tape would not adhere to rabbit's skin with sufficient firmness to maintain the desired tension.

In Rabbit 4, two incisions about 2 cm. in length were made, one at either side of the midline of the back. In the first, the natural tendency of the skin edges to gape was exaggerated by sutures placed at either side of the wound with the tension applied at right angles to the line of the incision. In the second, the sutures were placed beyond the ends of the incision with tension applied in the line of the cut. 4 days later, the wounds were excised and sectioned. Examination of the

and to the line of last tension. While no cross fibroblasts were found in the scar line, it seemed possible that this result might be interpreted as indicating merely that the majority of the fibroblasts had been laid down after the shift in tension was made, and had obscured the few laid down earlier. In consequence, in a repetition of the experiment, the interval between the shift in tension and the excision of tissue was cut down from 4 days to 2. In sections from this tissue, while the general direction of the fibroblasts in the scar was parallel to the line of incision and of the last tension, yet many lay in a direction oblique to this line, and the bodies of others were definitely curved as if the cell were in the act of turning. This seemed to clear up any question that might have persisted concerning the result of the earlier experiment.

CONCLUSION.

These experiments seem to justify the conclusion that mechanical tension may determine the polarity of cell division and line of growth of developing fibroblasts, and further that a shift in tension may cause a shift in position of fibroblasts already formed.

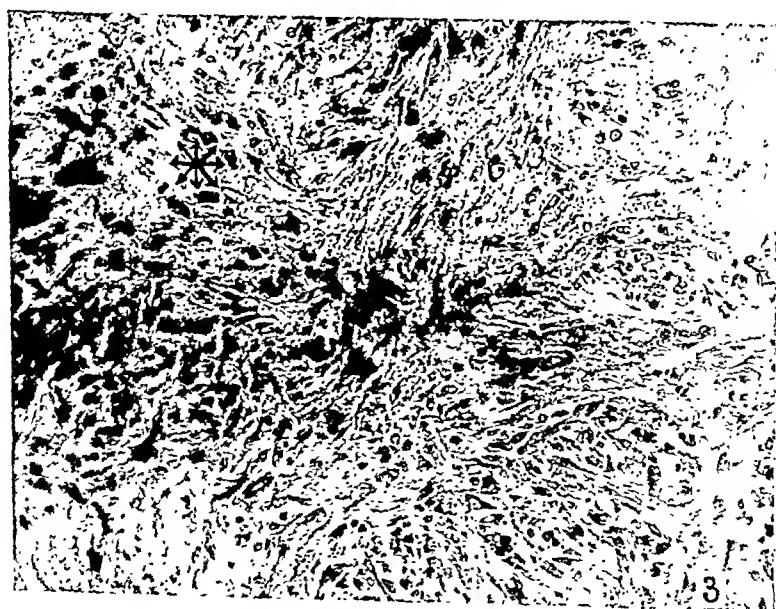
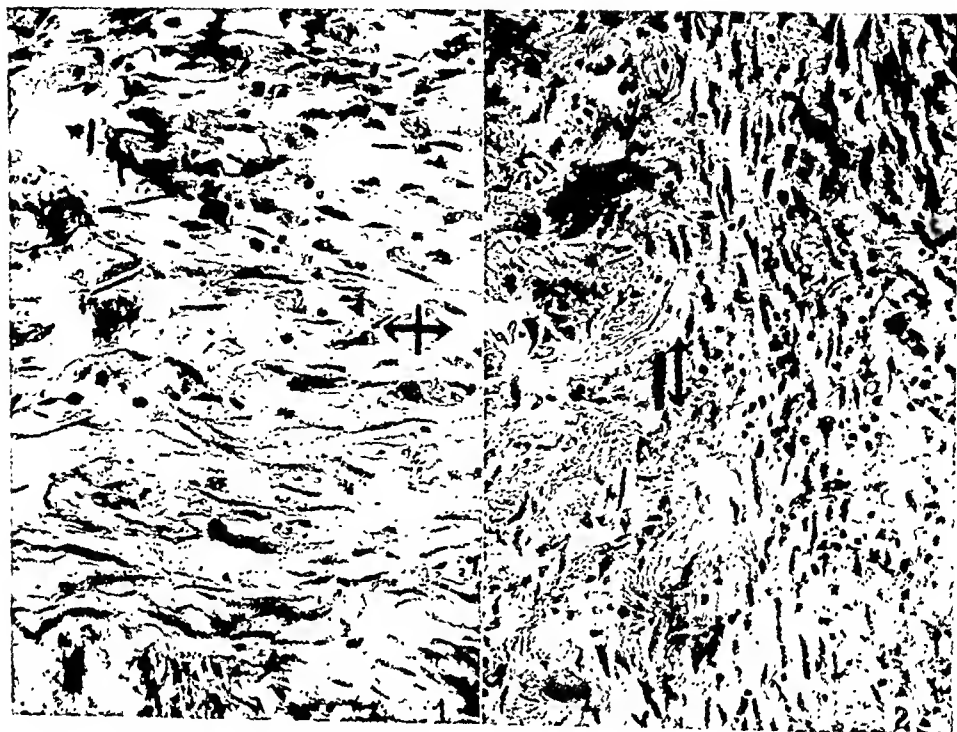
EXPLANATION OF PLATE 4.

The figures are photomicrographs of healing wounds, and were made from sections cut in a plane parallel to the skin surface.

FIG. 1. Section of healing wound in which tension was made perpendicular to line of incision which is vertical in the illustration.

FIG. 2. Section of healing wound in which tension is parallel to the line of incision, which is likewise vertical in the illustration.

FIG. 3. Section of knife point incision with radial centrifugal tension.



(Bunting and Eades: Polarity of growing fibroblasts.)

Babkin⁵ this observation was confirmed by Klodnizki⁶ and also by Boldyreff.⁷

The demonstration of the fact that bile need not enter the intestine save at long intervals of time has made it a matter of greater interest to study the conditions under which the escape of bile to the gut actually does take place. Though much work has already been done upon the theme⁸ there is little information to be had that is not based on indirect methods of observation; and there is but slight accord among workers upon the problem.

In the present paper the alterations occurring under pathological circumstances in the resistance to the flow of bile into the intestine will be described. The observations were made upon healthy, unanesthetized animals. In a paper to follow, the influence of the gall bladder will be considered.

PREVIOUS LITERATURE.

Anatomically the sphincter of the common duct is a well defined structure. As far back as 1680, Glisson⁹ suggested its existence but nearly two centuries elapsed before other investigators took up its study. In 1878 Gage¹⁰ first described the presence of circular fibers surrounding the duct in the ampulla of Vater, but the sphincter is best known through the detailed work of Oddi.¹¹ In many animals and in man there is about the lower portion of the common duct a discrete ring of muscle, distinct from the surrounding duodenal musculature.¹²⁻¹⁴

The physiology of the musculature of the lower portion of the common duct has only recently received much attention.^{8,15} Oddi¹⁶ on the basis of his experi-

⁵ Babkin, B. P., *Die äussere Sekretion der Verdauungsdrüsen*, Berlin, 1914, 338 *et seq.*

⁶ Klodnizki, Dissertation, St. Petersburg, 1902, cited by Babkin,⁵ p. 341.

⁷ Boldyreff, W. H., Dissertation, St. Petersburg, 1904, cited by Babkin,⁵ p. 341.

⁸ Mann, F. C., *Physiol. Rev.*, 1924, iv, 251.

⁹ Glisson, F., *Anatomia hepatis*, 1681, quoted by Oddi.¹¹

¹⁰ Gage, S. H., *Am. Quart. Micr. J.*, 1878-79, i, 123.

¹¹ Oddi, R., *Arch. ital. biol.*, 1887, viii, 317.

¹² Hendrickson, W. F., *Bull. Johns Hopkins Hosp.*, 1898, ix, 221.

¹³ Helly, *Arch. mikr. Anat.*, 1899, liv, 614.

¹⁴ Mann, F. C., *J. Lab. and Clin. Med.*, 1919-20, v, 107.

¹⁵ Westphal, K., *Klin. Woch.*, 1924, iii, 1105.

¹⁶ Oddi, R., *Sperimentale*, 1894, xlviii, 180, as abstracted in *Schmidt's Jahrb.*, 1895, cccxv, 120.

THE PHYSIOLOGICAL VARIATIONS IN RESISTANCE TO BILE FLOW TO THE INTESTINE.

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PLATE 5.

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The flow of bile, from its source within the liver to its issue into the duodenum, is greatly influenced by two structures situated along the path of flow. These, the musculature about the lower portion of the common duct and the gall bladder, not only effect the actual movement of bile but by their combined activities modify the character of the secretion as well. The common bile duct, after entering the wall of the duodenum, passes for a considerable distance through its muscular coat to enter the intestinal lumen at the ampulla of Vater. At this terminal portion is situated the sphincter of Oddi. This structure, aided perhaps by the musculature of the duodenum surrounding the lower portion of the common duct, exerts a pressure sufficient to maintain closure of the orifice of the duct during much of the time. Under these conditions the direction of the flow of bile is not continuously from the liver to the intestine but more often in the reverse direction, from the liver back into the gall bladder. Here, as has been shown in this laboratory,¹ the bile is concentrated with remarkable rapidity. The gall bladder is thus enabled to admit and store the bile coming to it during the usual digestive interim. Nearly 30 years ago Bruno,^{2,3} working with "Pavlov biliary fistula" dogs,⁴ noted that bile emerged from the intact ampulla only at long intervals of $1\frac{1}{2}$ to $2\frac{1}{4}$ hours, and in small amounts during fasting periods. According to

¹ Rous, P., and McMaster, P. D., *J. Exp. Med.*, 1921, xxxiv, 47.

² Bruno, G. G., *Arch. sc. biol.*, 1899, vii, 87.

³ Bruno, G. G., Dissertation, St. Petersburg, 1898, cited by Babkin.⁵

⁴ Pavlov, J. P., *Ergebn. Physiol.*, 1902, i, 1. Abt., 246.

discomfort. No such observations on the unanesthetized animal have been made in the past. The above mentioned observations by workers in Pavlov's laboratory^{2,6,7} were indirect in nature and were frequently complicated by difficulties inherent in the procedure. Under the circumstances of the Pavlov biliary fistula⁴ the portion of the duodenal mucosa transplanted to the body surface and containing the sphincter undergoes a gradual atrophy and retraction. The biliary tract and especially the portion about the ampulla becomes infected and the flow of bile is frequently obstructed.

Technique.

Normal, quiet, healthy dogs were employed in which the common bile duct was intubated, under ether, in two directions, toward the liver and toward the ampulla of Vater, the duct being cut between.²⁴ The gall bladder was excluded by section of the cystic duct between ligatures. A diagram of this scheme of intubation, "altercursive intubation," has already been published.²⁴ By means of it the bile is brought to the surface and can either be collected there or turned back into the common duct at will to flow as usual through the ampulla of Vater into the duodenum.

In Fig. 1 is shown a dog with the "altercursive intubation" installed, but with the protective wrappings removed from the joints of the tubing. The tube nearer the animal's head leads to the cannula that receives bile from the liver, the other connects with the cannula directed toward the ampulla of Vater. The excellent healing about the tubes should be noted. By means of a clamp placed on the tubing one can direct the flow of bile into the bag for collection, or prevent it from entering with result that it returns to the common duct at a point not over $\frac{1}{2}$ cm. from where it left it. As the figure shows, the rubber balloon is supported in a light wicker basket which is held in place against the animal's side by adhesive strips. This in turn is covered by a muslin and canvas jacket which laces up the back and, while permitting freedom of motion to the animal, prevents it from dislodging the apparatus. Sterility of the tube system is maintained by collecting the bile under aseptic precautions.

During the manipulations incident to the experiments sterility of the system can be maintained only by the most scrupulous care. Manometers and tubes were ster-

²⁴ McMaster, P. D., and Elman, R., *J. Exp. Med.*, 1925, xli, 513.

ments claimed for the sphincter nervous control independent of that of the neighboring intestinal muscle, further describing a center for it in the spinal cord opposite the first lumbar vertebra. He measured the sphincter tonus by observing the height of a column of water supported in a tube tied in the common duct. Doyon determined in anesthetized animals the amount of fluid passing through the ampulla from a specially constructed manometer connected with the common duct.¹⁷ He claimed for the sphincter a reaction on reflex stimulation only, with contraction upon irritation of the gastric mucosa with acetic acid and relaxation following electrical stimulation of the central end of the cut vagus or upon section of the medulla. He quoted the observation of Vulpian, that after "*piqûre*" of the medulla in rabbits, the duodenum is found, at autopsy, filled with dark brown bile.¹⁸ Reach¹⁹ studied the resistance offered to the passage of bile through the ampulla in a way similar to that of Doyon but used the completely isolated common duct immersed in warm Ringer solution. Bruno,² Klodnizki,⁶ Boldyreff,⁷ and much later Rost,²⁰ employing unanesthetized dogs with a duodenal fistula, observed intermittent gushes of bile from the ampulla, under a variety of circumstances, and deemed them indicative of a relaxation of the sphincter. These findings will be discussed in detail further on, as too will the recent work of Cole,²¹ who found in anesthetized dogs a relationship between the resistance to the flow of bile through the lower common duct and the reaction of the gastric contents.

Recently the importance of the action of the sphincter of Oddi as apart from that of the duodenal musculature surrounding the lower end of the common duct, has been called in question.^{22,23}

In the present work we have employed as a criterion of the activity of the musculature about the lower portion of the common duct the pressure necessary to force bile past it into the intestine. No attempt has been made to differentiate the activity of the duodenal musculature in this region from that of the sphincter. The circumstances of the observations have been especially favorable, involving as they have natural conditions in healthy, robust animals, with no disturbance of the biliary tract other than that incident to an intubation of the common duct. The pressure determinations, made long after operation, required no anesthetic since they produced not the slightest

¹⁷ Doyon, M., *Arch. physiol. norm. et path.*, 1894, vi, series 5, 19.

¹⁸ Doyon, M., *Arch. physiol. norm. et path.*, 1893, v, series 5, 683.

¹⁹ Reach, F., *Zentr. Physiol.*, 1912-13, xxvi, 1318.

²⁰ Rost, F., *Mitt. Grenzgeb. Med. u. Chir.*, 1913, xxvi, 711.

²¹ Cole, W. H., *Am. J. Physiol.*, 1925, lxxii, 39.

²² Carlson, A. J., *J. Am. Med. Assn.*, 1925, lxxxv, 1468.

²³ Burget, G. E., *Am. J. Physiol.*, 1925, lxxiv, 583.

given at stated times and the measurements made in relation to these times. The food consisted in every case of a liberal mixture of lean meat and bread soaked in milk, 450 gm. of the former to each kilo of the latter. The dogs were allowed to eat until satiated and the food was then removed. Special attention was paid to the influence of incidental body movements of the animals during the observations. In most cases the dog stood quietly for long periods with only occasional turning movements of the body, as it looked from one part of the room to the other. Since many duplicate observations were made at one time the influence of such body movements could be tested. It was found to be very slight.

The Normal Resistance to the Flow of Bile into the Intestine.

In the past numerous workers have measured how high a column of water had to be in a tube connected with the common duct in order to start a flow through the common duct. Widely divergent values ranging from 80 mm. to 600 mm. have been quoted.⁸ The workers mentioned made their measurements on the anesthetized dog, however, and they had also to reckon with the influence of trauma incident to a laparotomy. No mention has been made in many of the studies of any time relation to the giving of food.

We have tested the resistance offered to the flow of bile through the lower common duct in eighteen dogs appropriately intubated. A value for the "normal" pressure was found that was uniform within narrow limits though subject to alterations from feeding and digestion. In scores of experiments on sixteen animals it was a regular finding that the column of bile in the manometer connected with the lower common duct had to be raised to a level 100 to 120 mm. above it before flow started. This always ceased when the column had fallen to the 80 or 90 mm. level. The values were obtained between 4 and 12 hours after feedings.

The Increase in the Resistance to the Flow of Bile into the Intestine during Fasting.

Fasting for 24 to 72 hours results in a great increase in the resistance offered at the lower portion of the common duct to the flow of bile

ilized before use, and all joints protected with gauze sponges soaked in 5 per cent carbolic acid. Routine cultures of the bile and examinations of stained sediment from centrifuged specimens were made to discover the presence of organisms. Despite all precautions infection frequently occurred, not through the bile but as result of the repeated opening of the tube system to connect it with the manometer. Usually saprophytes such as did not change the character of the bile in important ways were the invading organisms. Nevertheless when this occurred experiments with the animal were at once abandoned as was done also whenever any obstruction developed in the cannulas, even a temporary one. In the present paper we consider only results that were obtained during the maintenance of sterile and unobstructive conditions within the biliary tracts of animals.

For the purpose of determining the resistance to the flow of bile into the intestine the tube leading to the lower common duct was temporarily disconnected at intervals and attached under aseptic precautions to a manometer filled with the animal's own uninfected bile. While the tests were in progress the liver bile was allowed to flow either into the balloon or into a sterile flask. In a number of experiments (as in that of Text-fig. 8) the manometer was temporarily substituted for the collecting balloon, with result that the pressure existing within the tube-duct system was registered. The latter arrangement is shown in Fig. 2. Under these circumstances, too, the tube draining liver bile could be clamped temporarily, with result that only the resistance offered to the flow of bile into the intestine was measured.

After a few repetitions of the procedure, at most, the dogs learned to stand quietly beside the manometer, for hours even, and they evidently enjoyed the attention bestowed upon them. The level of the common duct above the table on which they stood was roughly estimated by external measurements and the zero point of the manometer set at this level. Many pressure readings were taken from each animal daily, over long periods of time, and in each individual the zero point of the manometer was placed at the same height above the table as in the first determinations. The actual level of the common duct was determined at autopsy. The error from the presumptive level was so slight as to be negligible.

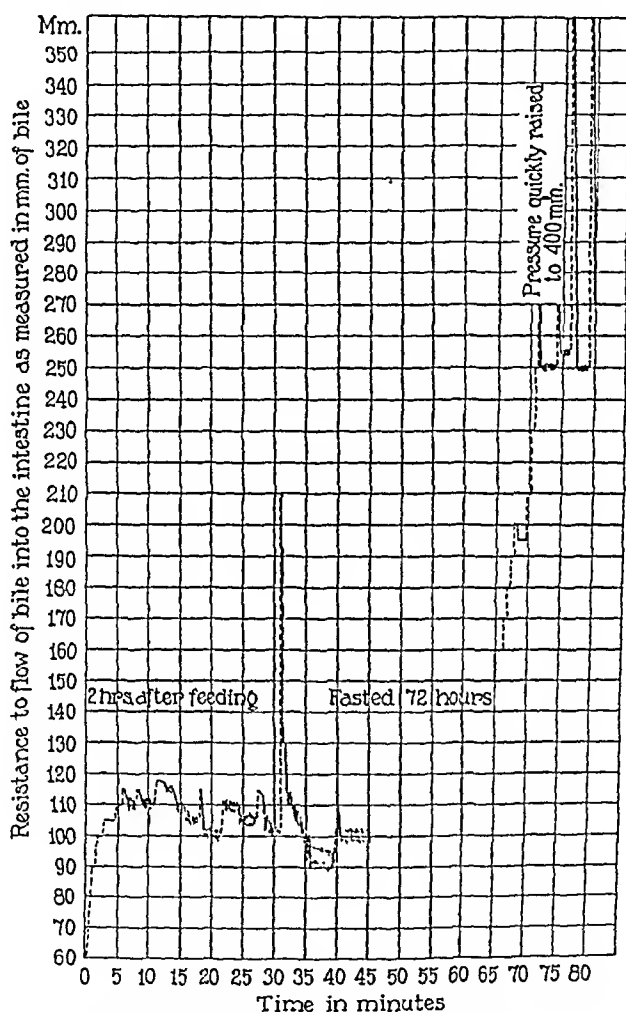
The resistance offered to the passage of bile into the intestine was tested by noting the height to which it was necessary to raise the column of bile or salt solution in the manometer, attached to the tube leading to the lower common duct, in order to start a flow. The height of this column when flow ceased was also noted. In one series of experiments we joined the lower common duct with a special manometer, to be described below, which was arranged to show the rate of bile flow into the intestine at a relatively constant pressure.

At the outset of these studies observations were made at random. Soon however an influence of the ingestion of food on the resistance offered within the lower common duct became evident to us. Thereafter, in the experiments which will now be described, feedings were

their object. Even when the bile column had been raised to the height of 400 mm., it fell back only to the 250 mm. level.

Dog 4, Text-fig. 2.—36 days after intubation the animal was healthy and active, following a fast of 48 hours.

11.00 a.m. Flow did not start until the column of bile in the manometer con-



TEXT-FIG 1. Influence of fasting on the resistance offered to the flow of bile into the intestine. See text.

nected with the lower common duct was raised to a level 180 to 190 mm. above it. Flow ceased when this had fallen to 150 mm. Twice thereafter the bile column was raised to 400 mm. but each time bile flow ceased when the pressure had fallen to about the 150 mm. level.

into the intestine. This finding was regularly obtained on twenty different occasions, in ten animals, that is to say in all of the tests made under the conditions. Whereas the pressure exerted by a column of bile 100 to 120 mm. in height usually sufficed to cause flow through the ampulla of Vater when the test was carried out 4 to 12 hours after a feeding, it was often necessary to raise the column to a height of 200 to 250 mm., to obtain this result in the fasting dog.

In relation to this phenomenon the findings of Bruno^{2,3} and Rost²⁰ have a suggestive interest. They observed unanesthetized dogs with Pavlov biliary fistulas and state that during fasting periods bile emerged from the ampulla only in small amounts and at long intervals of $1\frac{1}{2}$ to $2\frac{1}{4}$ hours.

Type experiments follow.

Dog 3, Text-Fig. 1.—At the time when the experiments were begun, 83 days after intubation, the animal was healthy and active. The resistance offered to the flow of bile through the lower common duct was tested 2 hours after a feeding by bringing the manometer and reservoir filled with the animal's own sterile bile into connection with the tube leading to the lower common duct.

In Text-fig. 1 and in the others to follow, the dotted lines indicate an artificial raising or lowering of the level of bile in the manometer, the continuous lines indicate changes in the pressure level consequent on physiological changes within the animal influencing the resistance to flow. Wherever a sinking of the bile column is depicted by a continuous line it is due to the flow of bile out of the manometer, through the lower common duct, into the intestine. As the figure shows, for Dog 3 the column of bile in the manometer was gradually raised and frequently allowed to remain, unaltered by the observer, during 1 minute or more. When it had been raised by him to 115 mm. spontaneous fallings of the column occurred, to the level of about 100 mm. After the tests had continued over half an hour, the column of bile in the manometer was abruptly raised to 200 mm. It fell almost as promptly to 100 and then more gradually to 90 mm. When the column of bile was now raised to 110 mm. there was a fall to 100 mm. as in previous tests of the sort.

Food was withheld for 72 hours, and after this the tests repeated. As the second portion of Text-fig. 1 shows, the column of bile in the manometer was raised gradually, with frequent pauses to give time for readjustment to take place on the part of the animal. Only when a pressure of 270 mm. had been reached did any flow into the intestine take place and this was checked when the pressure had fallen to 250 mm. Raising the column again to 270 mm. caused a drop to the 255 mm. level. Two attempts to cause lessening of the resistance to the flow of bile into the intestine by increasing the pressure considerably failed of

direct influence of feedings. Accordingly food was proffered to a fasting dog while a manometer was connected with the tube leading to the lower common duct. The recorded pressure had shown a great resistance to the passage of bile into the intestine, as always during a fast. At the mere perception of the food, before any had been given the animal, the column of bile suddenly dropped. This experiment was tried again and again in ten different animals and in over 75 per cent of the trials the result was the same, that is to say there was a relaxation of the musculature about the lower end of the common duct, due, most obviously, to a "psychic reflex." In these instances the actual act of eating often brought about a further relaxation as evidenced by a still greater drop of the bile column (Text-figs. 3 to 5). In all instances, including those in which no drop occurred at the perception of food, the act of eating regularly caused a sudden decrease in the resistance to the passage of bile into the intestine as shown by a drop of the column of bile in the manometer. Soon after this a somewhat increased resistance made its appearance. This will be discussed below.

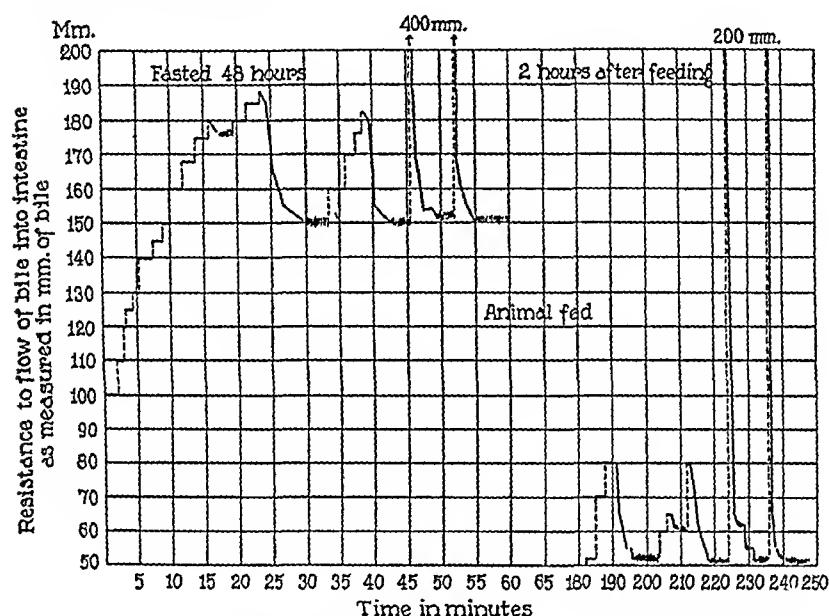
Usually but two or three experiments demonstrative of the psychic effect could be performed with one animal, because the mere act of placing it on a table for connection with the manometers brought about a conditioned reflex—the animal sniffed expectantly, became excited, and by the time the tubes could be connected to the manometer the change in resistance to bile flow which it was desired to study had already occurred,—a low column of bile now sufficed to cause flow through the ampulla.

What appears to be a phenomenon of similar nature was recorded years ago by Bruno² in Pavlov's laboratory.

Bruno worked with a dog in which a Pavlov biliary⁴ fistula had been made,—the ampulla of Vater surrounded by a good portion of duodenum had been transplanted into the anterior abdominal wall and the defect in the duodenum closed. On six occasions the animal was shown food but not allowed to eat, and on two of these occasions bile was seen to issue from the ampulla in amounts of 1.7 and 1.9 cc. respectively, though ordinarily the secretion did not appear until 15 to 45 minutes after the taking of food. Bruno also records the observation of Pavlov that gastric juice collected from fasting dogs during periods of "fictitious feedings" frequently contained appreciable amounts of bile. These instances of apparent sphincter relaxation are classed by Bruno among "psychophysiologic" reflexes.

12.00 noon. The dog was given food. 2 hours later a flow of bile into the intestine occurred repeatedly at the pressure exerted by an 80 mm. column of bile, and did not cease until the pressure had fallen almost to 50 mm. When the column of fluid in the manometer was raised to 200 mm. a fall followed and bile did not cease to pass into the intestine until the 50 mm. level had been reached.

Dog 5.—48 days after intubation the animal was healthy and active. 6 hours after feeding, 150 mm. of bile, in the manometer connected with the lower common duct, was sufficient to cause bile flow through the ampulla. This ceased when the level fell to 135 mm.

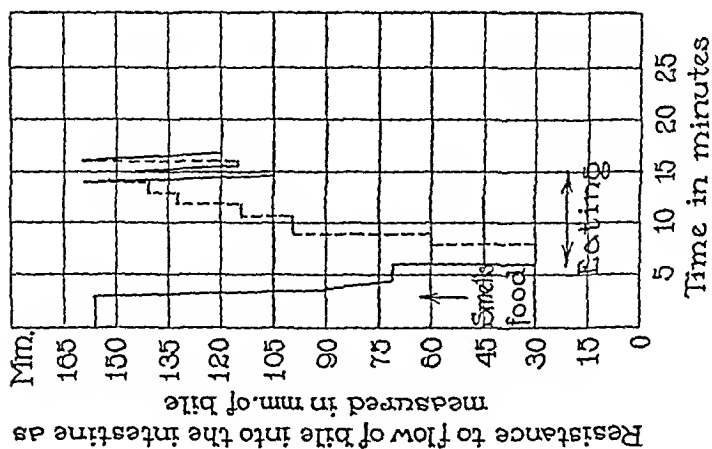


TEXT-FIG. 2. Effects of fasting and of feeding on the resistance offered to the flow of bile into the intestine.

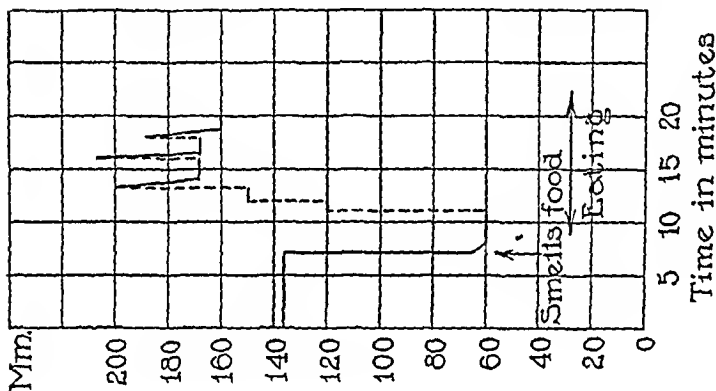
The dog was then fasted and 2 days later the tests repeated. A 240 mm. column of bile was needed to start flow. This ceased at the 200 mm. level.

Immediate Decrease in the Resistance to the Passage of Bile into the Intestine at the Perception of Food.

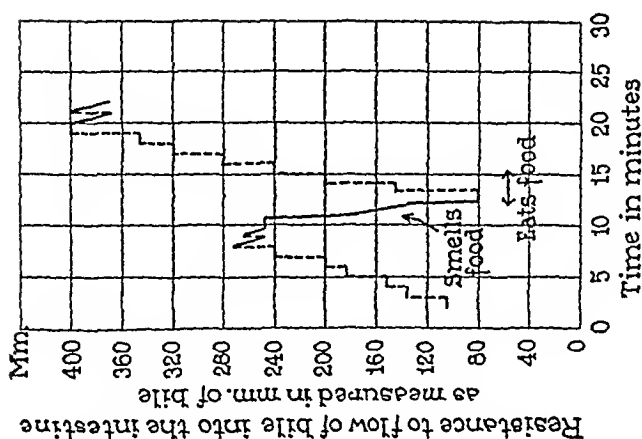
Increased resistance to the passage of bile into the intestine during a fast was a constant finding in all the experiments made upon ten different animals. It now became a matter of interest to study the



TEXT-FIG. 3.



TEXT-FIG. 4.



TEXT-FIG. 5.

TEXT-Figs. 3 to 5. Influence of the sight of food and feeding upon the resistance offered to the flow of bile into the intestine.

The phenomenon as we have observed it is represented graphically in Text-figs. 3 to 5. In Text-figs. 3 and 5 one can note as well the further lessening in resistance to the passage of bile into the duodenum when the animal began to eat.

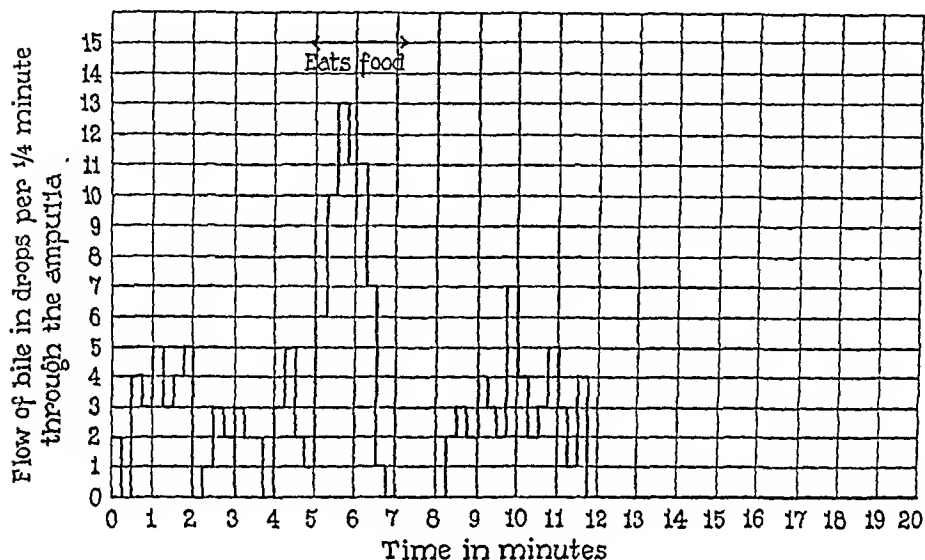
Dog 5, Text-Fig. 3.—55 days after operation the dog was healthy and active. Following a fast of 72 hours the resistance to bile flow was such as to uphold a 155 mm. column of bile in the manometer attached to the tube leading to the lower common duct. The animal was then shown food and at the first perception of it the column dropped immediately to 90 mm. and in $1\frac{1}{2}$ minutes to 70 mm. Eating was then allowed and at the first swallows the pressure fell at once still further, to 30 mm. The animal continued to eat. The column was raised within a period of 2 minutes to 60 mm. by allowing bile to flow into the manometer from the reservoir. No flow through the ampulla now occurred. The column was then raised to various levels as shown in the chart and allowed to remain 1 or 2 minutes at each. It did not fall until it reached the 160 mm. level. Following the initial decrease in the resistance offered to the flow of bile there had occurred almost immediately an increased resistance to it.

Another experiment on the same dog is represented in Text-fig. 4. It was performed 10 days later and after a fast of 48 hours. The result was similar but not as marked. The level of bile in the manometer connected to the lower common duct, and standing at 135 mm. above the latter, dropped to 60 mm. at the first perception of the nearness of food. No further change occurred with the actual taking of food but 2 minutes later while the animal was still eating, the column of bile in the manometer was raised to various levels as shown in the chart. Within 6 minutes it had been raised to 200 mm. Only then did flow of bile through the ampulla occur.

Dog 9, Text-Fig. 5.—35 days after operation the dog was healthy and active. Following a fast of 72 hours the resistance to the flow of bile into the intestine was tested as shown on the chart and found capable of supporting a 240 mm. column of bile. Food was then offered to the animal and immediately at the perception of it and before any had been eaten the bile column in the manometer dropped to 175 mm. and a minute later to 135 mm. Following the first swallows of food it fell further to 80 mm. After a minute of eating the column was raised by allowing bile to flow into the manometer from the sterile reservoir and after several such tests which disclosed a gradually increasing pressure the resistance was found to be high enough to hold back a column of bile 400 mm. in height.

The phenomenon was also demonstrated by measuring the amount of fluid passing through the ampulla of Vater at a constant pressure, using for such determinations a type of "flow" manometer. Any decrease in the resistance offered to the passage of fluid into the intestine

justed as to equal that of a column of bile 160 mm. high. There was a slight flow of about 50 drops (1.6 cc.) to the intestine before food was given, as the chart shows. But at the perception and taking of food there was a sudden and pronounced increase in this. Within 2 minutes three times as much fluid had entered the intestine as in the 2 minute period preceding the taking of food. Flow then ceased entirely. $1\frac{1}{2}$ minutes later there was a second period of flow lasting 4 minutes. In all 105 drops, or about 3.5 cc., of bile flowed into the intestine in the 7 minutes following the taking of food.



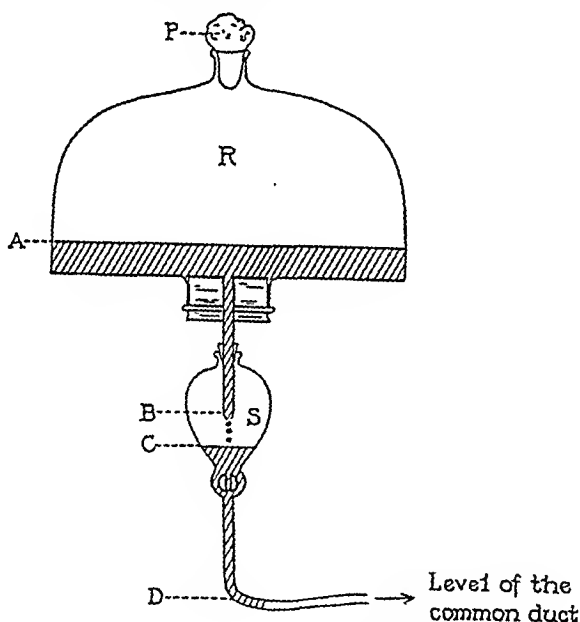
TEXT-FIG. 7. Influence of food ingestion on the flow of bile into the intestine.

The Secondary Increase and Decrease in the Resistance to the Flow of Bile into the Intestine after Feeding.

Following the transient decrease in the resistance to the passage of bile through the lower common duct after taking food, as described in these experiments, we observed a prompt increase in it as has just been noted. This was found by later experiments to endure for a variable time, becoming gradually less and less. Within half an hour or more the "normal" resistance was approached, namely that capable of supporting a column of bile 100 to 120 mm. in height. The increased resistance shortly after feeding has been illustrated in the experiments described above (Text-figs. 3, 4, 5, and 7). Babkin⁵ has reviewed the experiments with a Pavlov biliary fistula and has

resulted in a sudden increase in the rate at which the fluid flowed through the apparatus into the ducts.

The manometer used for this purpose is represented schematically in Text-fig. 6. A reservoir (*R*) was connected with a tube to an ordinary separatory funnel (*S*) which could be attached in turn to the tube leading to the lower common duct. The apparatus was sterilized by boiling or autoclaving. With all joints air-tight, the reservoir alone was open to the air but protected from contamination with a sterile cotton plug (*P*). By adding the heights of the two columns of fluid *A* to *B* plus *C* to *D* the pressure exerted by them could be estimated; and this

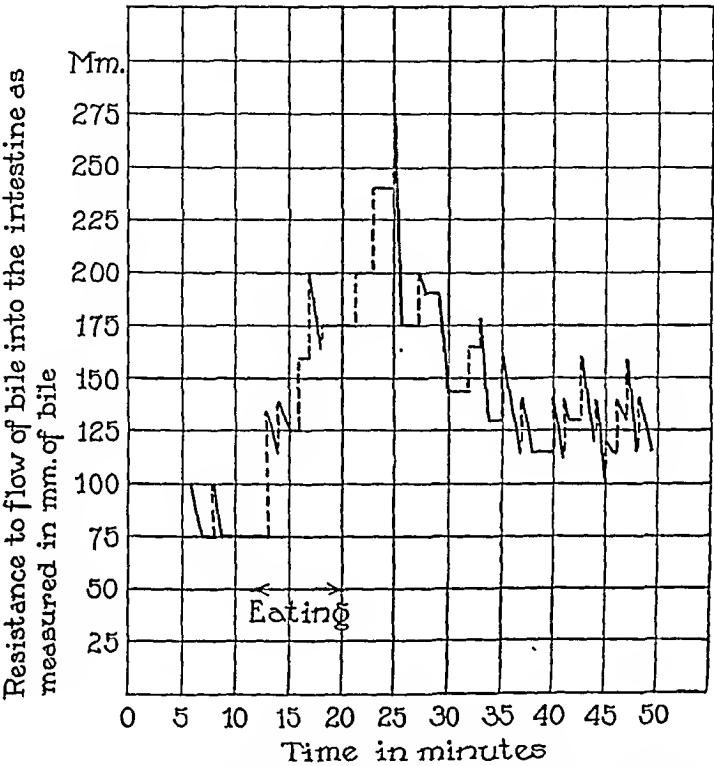


TEXT-FIG. 6. Diagram of the flow manometer.

could be varied at will by raising or lowering the apparatus. The rate of bile flow through the ampulla was measured roughly by counting the drops issuing from the nozzle *B* in quarter-minute intervals. Since 3 to 6 cc. of bile entered the intestine during the observations the surface level in the large reservoir was not appreciably changed, for a flow of 10 cc. reduced this but 1 or 2 mm.

Dog 19, Text-Fig. 7.—The operation for intubation of the common duct under ether had been performed 10 days before and the dog was healthy and active. After a fast of 48 hours the "flow" manometer was connected to the tube leading to the lower common duct and the pressure exerted by the apparatus was so ad-

intervals, the results of each being represented on the chart. The broken lines indicate artificial raisings of the pressure, the solid lines movements of the column of bile in the manometer in response to changes in the resistance.



TEXT-FIG. 8. The secondary increased resistance offered to the flow of bile into the intestine, following ingestion of food, and its gradual lessening, as shown by variations in the height of a column of bile in the manometer.

The Effect of the Ingestion of Weak Acid and Alkali upon the Resistance Offered to the Passage of Bile into the Intestine.

The reaction of the gastric contents may conceivably influence the resistance to the flow of bile into the intestine. For there is an immediate lessening in the resistance upon the taking of food, and a gradual increase in the half-hour or hour following, during which the reaction of the gastric contents is presumably becoming more acid. Since food may at this time be passing through the pylorus, a possible mechanical effect of peristalsis may in addition be invoked to explain the findings.

described a "latent period" between the giving of food and the first appearance of bile at the ampulla. The interval varied between 15 and 45 minutes depending upon the type of food given.

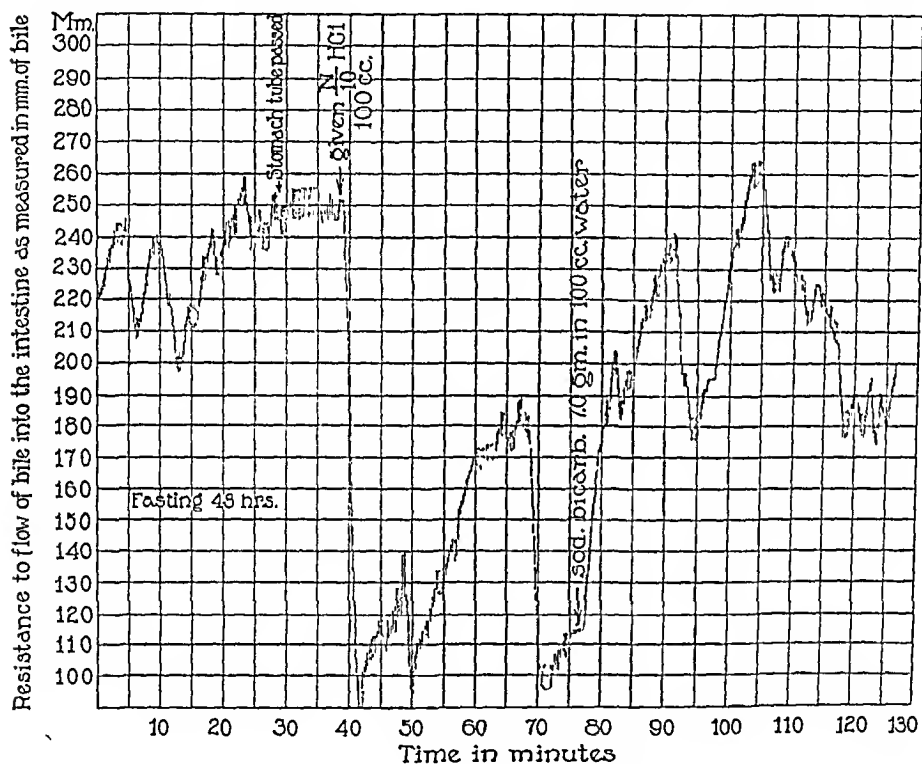
In our experiments the period of increased resistance in the lower common duct soon after feeding was of variable duration from 10 to 35 minutes. The degree of increase was often very great. In one case a 400 mm. column of bile was supported a few minutes after eating (Text-fig. 5). Eighteen feeding experiments in all were performed on ten dogs and the phenomenon was observed in every one. A type protocol will be given.

This experiment (see also Text-fig. 8) is chosen for description because it was carried out upon an animal fasted and then fed, several times previously, while the manometers were connected to the tube leading to the lower common duct. Through the experience of the repeated tests the animal had developed a conditioned reflex which resulted in the passage of bile into the intestine at a low pressure, but in spite of this a secondary increase in the resistance offered to passage regularly occurred after food was taken, and it became necessary to raise the column of bile in the manometer to a level of 275 mm. before fluid flowed into the intestine. Soon after this, however, relaxation of the musculature about the lower common duct began, as shown by the passage of bile through the ampulla at lower pressures.

Dog 9, Text-Fig. 8.—45 days after intubation the animal was healthy and active. It had been fed many times while manometer measurements upon it were under way. No food had been allowed for 48 hours previous to the present test. The animal became restless and sniffed about expectantly when placed on the table in order that the manometer might be connected to the tube leading to the lower common duct. It was not surprising, therefore, to find when this had been done, that a column of bile only 100 mm. in height was sufficient to cause a flow of bile through the ampulla, as the chart shows. The animal was shown food and permitted to eat. Within 2 minutes a 125 mm. column of bile was supported and in 10 minutes one of 240 mm. although a much lower column had caused flow into the intestine a few minutes before. Only after 20 minutes did the resistance decrease so that bile passed into the intestine at a lower pressure head, 175 mm. In half an hour the resistance had lessened to that originally noted, one supporting a column of about 100 mm. of bile; but even then it fluctuated. At one moment flow of bile into the intestine occurred at a pressure of 120 mm., and a little later only at one of 160 mm. Tests were made at short

pressive of the resistance had risen but only to 130 to 140 mm. another drop took place and again, 20 minutes later, when the manometer showed that the pressure in the biliary tract had risen to the equivalent of the weight of a column of bile 180 to 190 mm. in height, it once more, without obvious cause, fell rapidly to 100 mm.

Now 7.0 gm. of sodium bicarbonate in 100 cc. water was given. 15 minutes later the bile that was being secreted had raised the pressure in the biliary tract



TEXT-FIG. 9. Influence of the ingestion of acid and alkali upon the resistance offered to the flow of bile into the intestine.

to 230 mm., and 10 minutes later to 260 mm., when there once more occurred a flow into the intestine with a fall in pressure.

Control feedings of water elicited no changes in the resistance offered to the flow of bile into the intestine.

Experiments like this one, while not conclusive in themselves, point to the occurrence of changes in the resistance to the flow of bile through the common duct referable to alterations in the reaction of the gastric contents.

Fasting dogs were fed weakly acidified and alkalized solutions and the resistance offered to the passage of bile through the ampulla was estimated at such times. Very recently while these experiments were under way Cole²⁵ has reported observations of the sort on the anesthetized dog. He introduced acid or alkali into the stomach and observed an immediate decrease or increase respectively in the resistance to bile flow into the intestine as measured by the height of a column of fluid supported in a manometer tied in the common duct. We have noted the same relationship, though owing to the impossibility of controlling precisely the reaction of the gastric contents in the unanesthetized animal, our findings were not as constant as Cole's.

Seven experiments were performed in four animals appropriately intubated. Various amounts of $N/10$ HCl or 5 per cent $NaHCO_3$ were given to the fasting animals by stomach tube. A type experiment follows.

Dog 9, Text-Fig. 9.—18 days after an "altercursive intubation," the animal was healthy and active. No food had been given for the 48 hours previous to the experiment. An empty manometer was substituted for the balloon and, with the secretion of bile by the liver, a column of it 220 mm. high collected as a result of the resistance to passage of the secretion into the intestine. The findings after this level had been attained are charted in Text-fig. 9. They were recorded by the cooperation of two workers, one constantly observing and calling out the level of the bile column in the manometer, the other, who held a stop-watch, plotting as ordinates on graph paper the reported level with minutes as the abscissæ. The text-figure is the graphic representation of the findings obtained in this way, not a kymographic tracing, which it superficially resembles. The slight vertical fluctuations in the level of the bile column which will there be noted were due to respiratory movements. As the chart shows, a column of bile about 250 mm. in height could be maintained in the manometer. Slight decreases in the resistance occurred but the column did not fall below the 200 mm. level.

It has been estimated²⁵ that the acidity of gastric juice is equivalent to or slightly stronger than that of $N/10$ hydrochloric acid. Accordingly the animal was now given 100 cc. of $N/10$ HCl by a stomach tube already *in situ*. The tube had been passed 10 minutes previously without affecting the level of bile in the manometer. Practically at once, upon giving the acid, a drop of the bile column in the manometer to 90 mm. occurred. 10 minutes later when the pressure ex-

²⁵ Menten, cited by Mathews, A. P., *Physiological chemistry*, New York, 4th edition, 1925, 357.

which agrees well with many of the observations of others made under ether.⁸ In the fasting animal this resistance is always without exception much increased. However, at sight and smell of food it usually decreases and invariably does so at once upon eating, and, as our experiments show, permits an increased flow of fluid through the ampulla, at constant pressure. This marked but transient relaxation is followed almost as soon as food reaches the stomach by an increased resistance to the flow of bile which endures for about half an hour. Experiments with acid and alkali feeding show that the sequence of events just described with the exception of that due to the psychic stimulus may be explainable by changes in the gastric acidity after the taking and digestion of food. That this may not be the only factor engaged, and probably is not, is well recognized by us.

Whether the intermittent ejection of acid chyme through the pylorus stimulates relaxation of the musculature about the lower common duct cannot be determined from our observations. In favor of such a view is the finding that the resistance offered to bile flow through the ampulla during gastric digestion is a fluctuating one (see Text-fig. 8), but almost all our further evidence argues against it. For example, the lessening in the resistance to the passage of bile into the intestine which occurs when food is shown the animal takes place so promptly and rapidly that it must certainly be the result of a reflex. The same may be said of the rapid relaxation following administrations of acid to the fasting animal (Text-fig. 9). In the fluctuation of the resistance to the flow of bile through the ampulla during gastric digestion the duodenal musculature may well play a part.

A consideration of the rôle of the gall bladder in the passage of bile into the intestine is essential to any discussion of the physiology of bile flow. This will be taken up in a succeeding paper.

SUMMARY.

Under specially controlled conditions in the healthy unanesthetized dog we have measured the resistance offered to the flow of bile through the lower common duct.

The average resistance 4 to 12 hours after a feeding was found to correspond to the pressure of a column of bile 100 to 120 mm. in height.

DISCUSSION.

The work here reported is the first of this nature, so far as we are aware, that has been done upon animals under practically normal conditions.²⁶ Since the approach to the normal is of prime importance, procedures rendering the musculature of the lower portion of the common duct visible have been avoided. Under the circumstances of the experiments described it has been possible to measure the resistance offered to bile flow through the ampulla at any time desired. We have used this measurement as a criterion expressive of the conditions under which bile makes its escape into the intestines. No attempt has been made to differentiate experimentally the activities of the duodenal musculature about the lower common duct as apart from that of the sphincter of Oddi. Recently Carlson²² and Burget²³ have stressed the possible importance of the duodenal musculature in the retention or escape of bile. It undoubtedly plays a rôle especially during active peristalsis. More will be said of this below.

If the control of bile flow through the ampulla is due to the sphincter it might be objected that the operative interference incident to intubation of the common duct would probably destroy or at best interfere with nerve filaments passing to and from the muscle of Oddi. Since our findings were positive ones, the sphincter retaining its power of relaxation and contraction, such disturbances cannot have been very serious. Either the nerves affected play an unessential part in the function of the sphincter, or they had reestablished their normal connections.

Whatever be the explanation of our findings, they show definitely that the escape of bile into the duodenum is under a definite control and is markedly influenced by varying the physiological conditions.

In these experiments there was a remarkable constancy of the findings. In the normal unanesthetized animal recently fed the resistance to the passage of bile through the ampulla supports a column of bile or physiologic saline solution 100 to 120 mm. in height, a finding

²⁶ Since this was written, J. C. Potter and F. C. Mann have published in the *Am. J. Med. Sc.*, 1926, clxxi, 202, studies on the "Pressure changes in the biliary tract" in unanesthetized dogs, also under practically normal conditions. We regret that the appearance of this most interesting paper occurred too late to permit of discussion of their findings herein.

After a 24 to 72 hour fast the resistance was such as to support a much higher column of bile, one of 300 mm. at times. The exhibition of food to the fasting animal usually precipitated a reflex lessening in the resistance to the flow of bile to the intestine, the actual taking of food always brought it about. This was transient and was soon followed by a period of increased resistance lasting 10 to 30 minutes after food had entered the stomach. There succeeded a drop in resistance which was gradual and fluctuating.

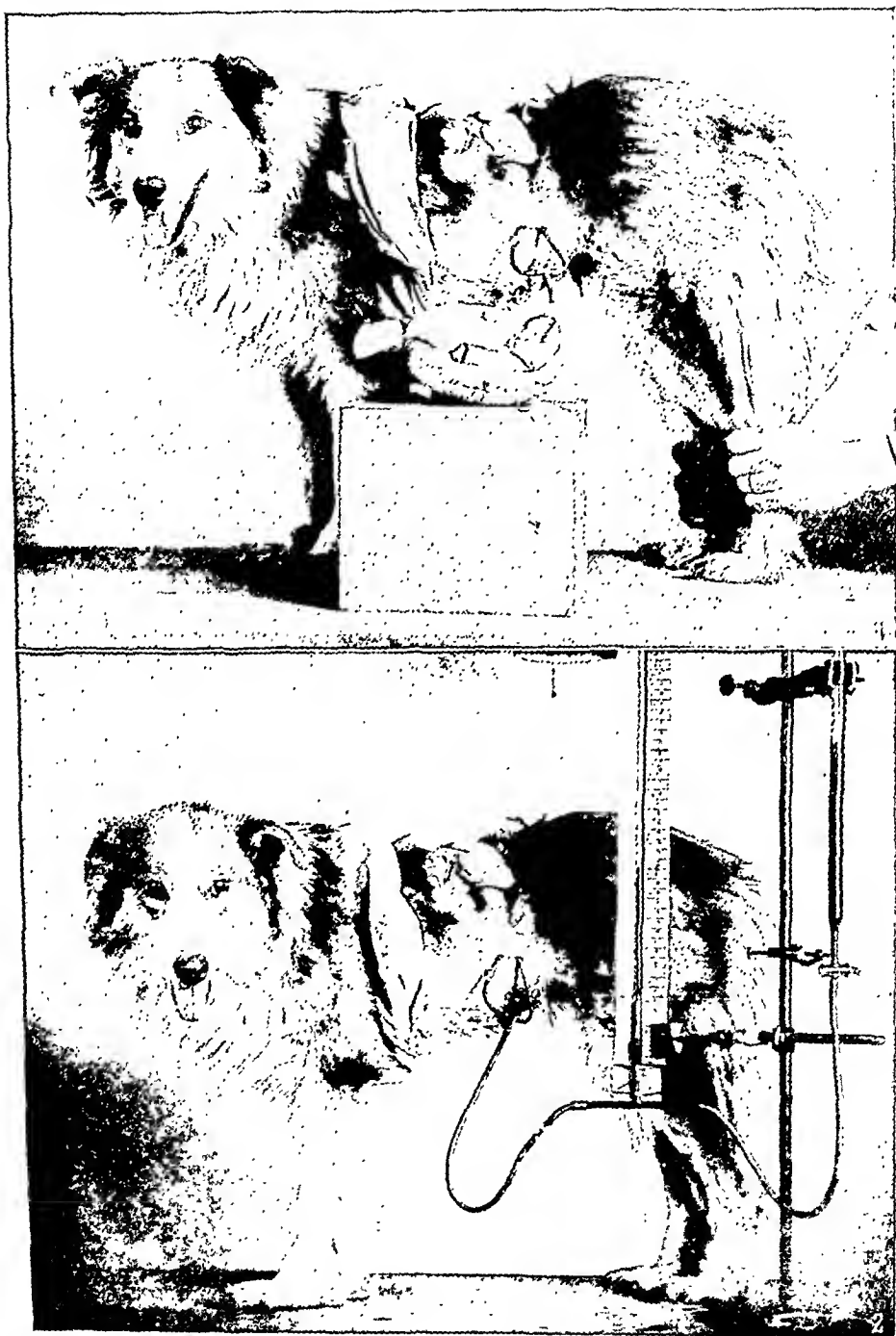
We have observed an increase in the resistance to the flow of bile into the intestine after alkali has entered the stomach, and a decrease after acid has been administered.

We attribute the sudden changes chiefly to the activity of the sphincter of Oddi.

EXPLANATION OF PLATE 5.

FIG. 1. Animal with an "altercursive intubation." For description see text.

FIG. 2. Manometer connected with the collecting tube of an "altercursive intubation." For description see text.



(Elman and McMaster: Resistance to bile flow to intestine.)

servers. Winkelstein,¹⁰ most recent among them, believes that the organ is purely passive, filling and emptying as result of extraneous alterations of the pressure brought to bear upon it, more especially in the course of respiration. That the gall bladder fills with bile is disputed by nobody.

Renewed interest in the problem of the contractility of the gall bladder has sprung from an application to it of the law of contrary innervation proposed by Meltzer.¹¹ He advanced an attractive hypothesis according to which, at each discharge of acid chyme from the stomach, the sphincter of Oddi relaxed and the gall bladder synchronously contracting, ejected a gush of bile into the duodenum. He noted in another connection that local applications of magnesium sulfate solutions to the duodenal mucosa cause relaxation of the muscles of the intestinal wall. The clinical application of this hypothesis and fact by Lyon¹² has resulted in many attempts at "non-surgical drainage of the gall bladder" with the accumulation of a great mass of conflicting evidence as to whether this object is ever accomplished. Since the literature has been reviewed by others¹³⁻¹⁵ we need not enter into it here.

In a companion paper to the present one¹ it has been shown that an immediate decrease in the resistance offered to the passage of bile into the intestine takes place when an animal begins to eat, and usually indeed upon the mere perception of food. Conditions at such times are especially favorable to the escape of bile into the intestine. Does this occur in quantity, and what are the forces promoting it? Our first experiments have been directed to these points.

Technique.

A full description of the technique used in our studies has been given in the accompanying paper.¹ We have utilized healthy dogs in which some time previously there was installed, under ether anesthesia, the apparatus of an "altercursive intubation"² whereby bile flowing from the liver can either be collected under sterile conditions or turned back at will into the common duct to flow as usual through the ampulla of Vater into the duodenum. In order to study the influence of the gall bladder, the connections of the organ were left

¹⁰ Winkelstein, A., *J. Am. Med. Assn.*, 1923, lxxx, 1748.

¹¹ Meltzer, S. S., *Am. J. Med. Sc.*, 1917, clii, 469.

¹² Lyon, B. B. V., *J. Am. Med. Assn.*, 1919, lxxiii, 980.

¹³ Mann, F. C., *Physiol. Rev.*, 1924, iv, 251.

¹⁴ Friedenwald, J., Martindale, J. W., and Kearney, F. X., *J. Med. Research*, 1922, ii, 349.

¹⁵ Matsuo, I., *J. Am. Med. Assn.*, 1924, lxxxiii, 1289.

ON THE EXPULSION OF BILE BY THE GALL BLADDER; AND A RECIPROCAL RELATIONSHIP WITH THE SPHINCTER ACTIVITY.

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In the present paper we shall offer proof that the gall bladder discharges bile on physiological occasion, through the agency of forceful contractions. The phenomenon has been encountered in healthy, unanesthetized animals with the biliary passages permanently intubated in such a way¹⁻³ that the pressure exerted by the vesica fellea and the resistance to the flow of bile into the gut could be studied simultaneously and under controlled conditions. The existence of a relationship between the activity of the gall bladder and that of the musculature about the lower common duct, as expressed in alterations of the resistance just mentioned, has been demonstrated by the method.

The most diverse views of gall bladder activity and function have been proposed in the past, and they all still find adherents. Some workers hold the gall bladder to be an active, contractile viscus,⁴⁻⁸ capable of expelling its contents. In contrast to such an opinion Sweet,⁹—who reasons from the anatomic structure of the human viscus and cystic duct,—believes that nothing entering the gall bladder through the duct ever leaves it by the same route but is sooner or later resorbed. The midground between these positions is held by many ob-

¹ Elman, R., and McMaster, P. D., *J. Exp. Med.*, 1926, xliv, 151.

² McMaster, P. D., and Elman, R., *J. Exp. Med.*, 1925, xli, 513.

³ Rous, P., and McMaster, P. D., *J. Exp. Med.*, 1923, xxxvii, 11.

⁴ Doyon, M., *Arch. physiol. norm. et path.*, 1893, v, series 5, 678, 710.

⁵ Bainbridge, F. A., and Dale, H. H., *J. Physiol.*, 1905, xxxiii, 138.

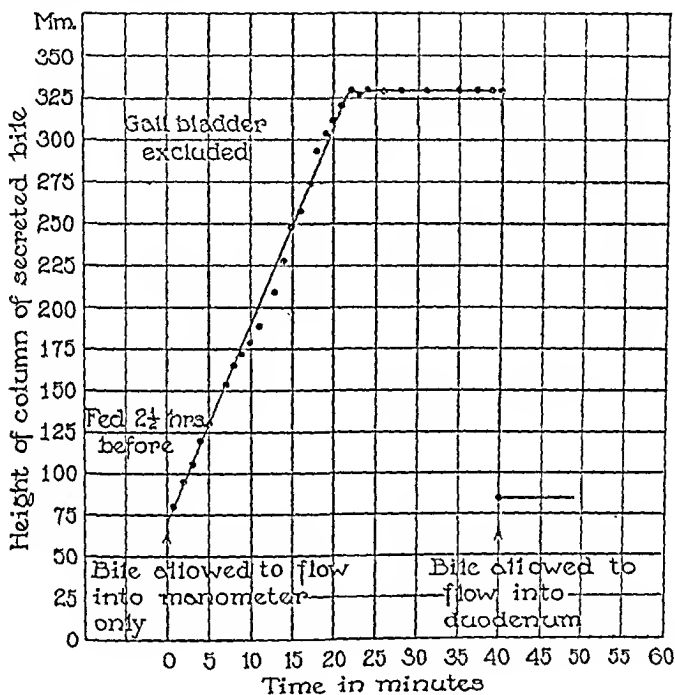
⁶ Okada, S., *J. Physiol.*, 1915-16, i, 42.

⁷ Judd, E. S., and Mann, F. C., *Surg., Gynec. and Obst.*, 1917, xxiv, 437.

⁸ Mann, F. C., and Giordano, H. S., *Arch. Surg.*, 1923, vi, 1.

⁹ Sweet, J., *Internat. Clin.*, 1924, i, 187.

and Simpson,¹⁶ who found in dogs under chloroform anesthesia with the gall bladder excluded, that the bile filled a manometer, to an average maximum of 300 mm. when obstruction was produced. The animals were fed 6 hours before the experiment. The time required for the secretion to reach the high level was variable, from 1 or 2 to often as much as 4 hours. Mitchel and Stifel¹⁷ found in



TEXT-FIG. 1. The maximum secretion pressure of bile and its rate of formation in the presence of a pressure obstacle. The dots record the actual observations on an animal. It will be seen that the rate of secretion was unaffected by the increasing pressure obstacle.

the bile ducts of cats anesthetized after total biliary obstruction of 2 $\frac{1}{2}$ to 6 days duration a pressure equivalent to 250 to 300 mm. of water.

It seemed advisable to determine the pressure developing on

¹⁶ Herring, P. T., and Simpson, S., *Proc. Roy. Soc. London, Series B*, 1907, lxxix, 517.

¹⁷ Mitchel, W. T., and Stifel, R. E., *Bull. Johns Hopkins Hosp.*, 1916, xxvii, 78.

intact in a number of animals, with result that the cannula inserted into the upper part of the common duct formed the means of exit for both liver and gall bladder bile; while in others the cystic duct was sectioned and ligatured at the time of the intubation. In some cases the gall bladder influence was studied more directly by intubation of the cystic duct or of the duct which results from the union of this latter with the small tributary from the left central lobe of the liver. As an "altercursive intubation" of the common duct had also been carried out in these cases, they were the subject of what one may term a "triple intubation," the details of which will be described more fully below.

As in our many previous intubations of various sorts aseptic precautions were taken in the handling of the tubes. Only instances in which the bile and biliary tracts remained sterile will be considered in this paper. Cultures of the bile were taken at frequent intervals during the course of the observations and stained sediments from it, as obtained by centrifugation, were examined for bacteria. At autopsy the sterility of the bile was again ascertained by culture. Cultures from the liver tissue were also made on agar and in bouillon. Despite the most careful precautions the bile of many of our animals became infected owing to the constant handling of the tubes. All such instances were discarded as, too, were any suggestive of obstruction in the cannula-tube systems.

The animals were given a diet of bread wet with milk, and lean meat, 450 gm. of the latter to each kilo of the bread and milk. They were fed daily in the morning, and allowed to eat until satiated. The time when the animal was last fed proved to be an important factor in the results and careful record was made of it.

To determine the pressure exerted upon the contents of the gall bladder and bile ducts, and to measure resistance to the flow of bile into the intestine, the rubber tubes leading from and to various regions of the biliary tract were repeatedly brought into connection with sterile manometers previously filled with the animal's own sterile bile as collected some time previously. The changes in the level of the columns of bile in the manometers were recorded by the cooperation of two or four observers, according to the number of manometers to be observed. The watchers at each of these instruments read off all the alterations in levels of the bile columns in the manometers as they occurred, while the other workers, who held stop-watches, plotted, on graph paper, the pressures as ordinates against time in minutes as abscissæ. Text-figs. 1, 2, 5, and 6 represent these graphs as obtained.

Secretion Pressure of the Bile.

Before the action of the gall bladder can be intelligently discussed it is necessary to consider the cause of the pressure existing within the biliary tract. Of the forces producing it, that of bile secretion by the liver is chief. This has been well studied, notably by Herring

The Effect of Food on the Flow of Bile in Animals with Gall Bladder Excluded.

Although changes in the pressure exerted against bile secretion do not affect its rate of formation, the giving of food does. This latter fact has long been a truism of physiology.^{19,20} Yet as a preamble to further observations, it has been necessary to study the phenomenon carefully in our dogs.

In eight animals with ligated and sectioned cystic duct, the tube draining liver bile was connected to a sterile graduate and the amounts of bile secreted during 5 minute periods were recorded. While the bile was being collected the animals were allowed to eat the bread, milk, and meat mixture for 2½ to 3 minutes. No change in the rate of secretion was noted during the first 5 to 15 minutes. Then a gradual progressive increase appeared, as depicted in the lower portions of Text-figs. 5 and 6. The stimulus was short lived, the amount of bile secreted returning to "normal" in about 45 minutes. The bile was not markedly changed in its gross appearance at any time—it was still limpid, and a rather light brown in color—but it usually was found on analysis to contain less pigment per unit of volume than before the feeding, in conformity with previous knowledge.^{21,22} When, however, the experiment was repeated in animals with the gall bladder connections undisturbed, the bile obtained after feeding was very different from that voided previously. Before this latter finding can be discussed in detail we must digress to consider certain pressure relations existing within the biliary tract.

The Filling of the Gall Bladder and Retention of Its Contents.

In an earlier paper from this laboratory²³ it was shown that the pressure exerted by a column of bile 60 to 70 mm. high connected with the common duct is sufficient to cause a flow of bile into the gall bladder of the anesthetized dog. In the unanesthetized animal the pressure within the viscus is generally about equal to that of a column

¹⁹ Bruno, G. G., Dissertation, St. Petersburg, 1898; and Klodnizki, Dissertation, St. Petersburg, 1902; cited by Babkin, B. P., *Die äussere Sekretion der Verdauungsdrüsen*, Berlin, 1914, 344.

²⁰ Bruno, G. G., *Arch. sc. biol.*, 1899, vii, 87.

²¹ McMaster, P. D., Broun, G. O., and Rous, P., *J. Exp. Med.*, 1923, xxxvii, 395.

²² Dastre, A., *Arch. physiol. norm. et path.*, 1890, ii, series 5, 800.

²³ Rous, P., and McMaster, P. D., *J. Exp. Med.*, 1921, xxxiv, 47.

obstruction in our unanesthetized animals. Accordingly, many days or weeks after intubation, the liver bile of dogs with gall bladder excluded by severance of the cystic duct was allowed to flow directly into a vertical manometer tube from which there was no escape except by way of the opening at the top. The fluid rose to a height slightly greater than that noted by Herring and Simpson in anesthetized animals, that is to say to about 320 mm. on the average, the figures for the individual animals, two of which were tested a second time, being as follows: 302,* 305, 308,* 314, 318, 321, 324, 326, 326,† 330, 333,† 335.¹⁸ The time required to reach the maximum point was much shorter than in the experience of these observers, being only 15 to 30 minutes. No relation was evident between the size of the animal and the pressure obtained.

In Text-fig. 1 is charted an instance typical of all. The animal yielding it had undergone an "altercursive intubation" 2 months before and was healthy and active. $2\frac{1}{2}$ hours before the experiment was begun food was given. The tube draining the liver bile, which up to that time had been delivering the fluid to the intestine by way of the rubber detour, was then brought into connection with a vertical glass manometer of 3 mm. bore. As the liver secreted bile the column rose rapidly to a level 330 mm. above that of the duct-cannula junction. It will be noted that despite the progressive pressure obstacle the rate of secretion remained constant, the bile column rising regularly, so that the readings of its height plotted against the time formed a straight line. The finding was obtained in all such experiments, twelve in number in ten different animals. The formation of bile, then, was as rapid against high pressures as low, until a maximum point was reached at which secretion suddenly ceased in the face of the high pressure.

When bile was collected at zero pressure for the brief periods necessitated by the experiments the same uniformity was noted in the rate of secretion from minute to minute. More will be said of this below.

¹⁸* and † indicate that the figures were obtained from the same animals upon different occasions.

prevent the development of a high pressure within the duct system. And such is the case.

In six animals an "altercursive intubation" was done without interference with the gall bladder connections. In such instances the bile simply made the detour of the tubes on its way to the duodenum, and encountered at the lower end of the common duct the normal resistance to its passage into the gut. This, from our figures given in the previous paper, would be sufficient, except after the taking of food, to keep the gall bladder full.

In nine experiments upon these animals with undisturbed gall bladders, we connected to a manometer the tube leading from the upper portion of the common duct, taking due care that no bile escaped from the latter during the process. The animals had been fed the usual meal of bread, milk, and lean meat several hours previously. During the succeeding several hours the column of bile never rose to more than 100 or 150 mm. above the level of the duct, that is to say it never more than approximately equalled the resistance which would normally have been exerted against the escape of bile into the intestine. In none of our experiments did the pressure within the common duct ever approach the maximum secretion pressure of the liver.

In Text-fig. 2 is plotted the result of such an experiment. In sharp contrast to this are the findings already described above (Text-fig. 1) when the gall bladder had been cut off from connection with the duct-manometer system.

Dog 12, 9½ kilos, Text-fig. 2. An "altercursive intubation" had been carried out 9 days previously, with the gall bladder connections left intact. The animal had been fed each morning and at the time of the observation was in the best of health. 2½ hours after feeding, the experiment was begun, the tube leading from the upper portion of the common duct being then connected with a manometer of about 3 mm. bore, one that is to say which required only 1 cc. of bile for a rise of 141 mm. in the column. As the chart shows, the pressure that developed and was maintained in the common duct during the next 4 hours equalled that of a column of bile about 125 mm. in height. There were frequently repeated, rapid but slight, incidental fluctuations due to the respiratory movements. Usually the column then rose or fell about 10 to 15 mm., occasionally on a deep breath 20 to 30 mm. The amplitude of the larger of these fluctuations and of certain more considerable yet still slight excursions of the

of bile 100 mm. in height, as our experiments to be detailed below will show. The secretion pressure being much more than this, it is obvious that no force other than that resulting from the liver activity is needed to fill the gall bladder, provided bile does not escape into the intestine. However, in our companion paper¹ several factors have been recognized which may conceivably be responsible for such an escape of bile through the alterations they excite in the resistance offered to bile flow by the musculature about the lower portion of the common duct. This resistance in the normal animal, fed 4 to 12 hours previously, is sufficient to support a column of bile 100 to 120 mm. in height. In the fasting animal, however, it will support one 250 to 300 mm. high. Immediately after taking food and again later, during the process of digestion, the resistance is greatly lowered, and a bile column 50 to 80 mm. in height suffices to cause a flow of bile through the ampulla. From this it is evident that save at these latter times the bile, as secreted, backs up perforce into the gall bladder, this being the way of least resistance. Whether there is an aspirating effect on the organ due to the respiratory movements, as suggested by Winkelstein,¹⁰ it is not necessary to consider, for the foregoing facts alone will suffice to explain the filling of the organ. Once within the gall bladder the bile undergoes rapid concentration.²² This concentrating activity may well be more important for the retention of the gall bladder contents than the valve-like reduplications of the mucous lining of the cystic duct (Heister's valves). What part these may play remains to be determined as does that of the circular ring of muscle sometimes situated in the cystic duct.²⁴

The Influence of the Gall Bladder on the Pressure of Bile in the Common Duct.

From the circumstance that the gall bladder receives and concentrates large amounts of bile, as well as because of the delay which its presence causes in the development of jaundice after obstruction of the common duct,²⁵ it might be inferred that the organ acts to

²⁴ Westphal, K., *Klin. Woch.*, 1924, iii, 1105.

²⁵ Afanassiew, M., *Z. klin. Med.*, 1883, vi, 281.

no evidence of an increase in pressure within the biliary tract above 125 mm., the level "normal" for the animal; and the experiment was discontinued.

Unlike the "straight line curve" of increasing pressure upon obstruction, obtained in animals with the gall bladder excluded, as noted in Text-fig. 1, no rise in the curve occurred during the first 4 hours when the gall bladder was present.

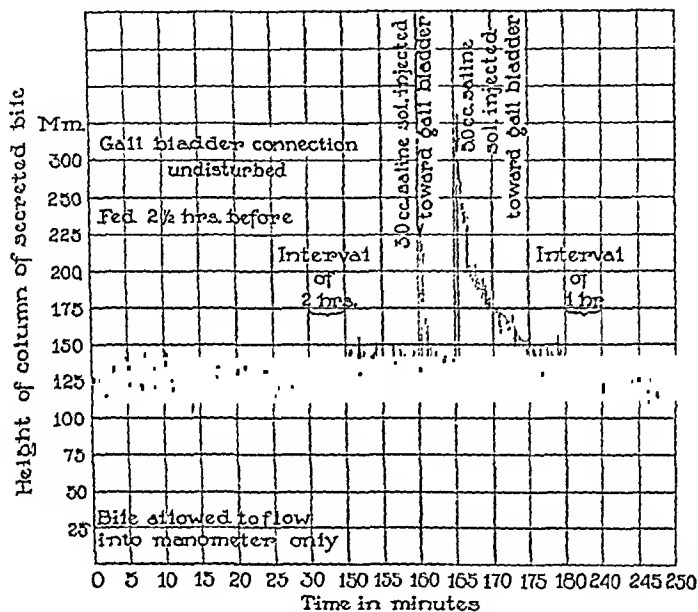
The Effect of Food on the Bile Yield of Animals with Gall Bladder Undisturbed.

In our dogs possessing intact gall bladders a decided difference was to be noted in the gross appearance of the bile collected before and after feeding, as already has been said. It may be recalled that the "altercursive intubation" in these animals permitted the bile simply to make a detour on the way to the gut. The normal resistance to the passage of bile into the intestine would be sufficient to divert the secretion to the gall bladder soon after a feeding.

The bile secreted by these animals when they had been fasting for 24 to 48 hours was allowed to drain freely into sterile graduates and then, after half an hour, food was given while the bile collection was continued. The secretion of these animals while fasting showed the characteristics of liver bile under such circumstances, that is to say it was more concentrated than in full fed animals; but immediately after the first swallows of food an abundant gush of far darker and more viscid bile suddenly flowed into the graduate, the darkness in color being due, as quantitation showed, to a high concentration of bilirubin. This experiment was performed twice with each of the six dogs with undisturbed gall bladder connections, and in all instances the same result was obtained. The phenomenon suggests, of course, a discharge of gall bladder contents. The finding (Text-fig. 3) should be compared with the delayed, and slow and gradual increase in the flow of bile observed after a feeding in animals with the gall bladder excluded from the collecting system (lower portions of Text-figs. 5 and 6). In instances of the latter sort in which liver bile alone is collected the food stimulus to secretion, far from causing the bile to become thicker and darker has effect to render it more abundant, more watery, and light in color,—a fact long known.

bile column in the manometer is shown in the text-figure which is typical of all such experiments. Only the readings of two brief periods each of half an hour and another of 10 minutes are recorded in the chart. As already stated the manometer remained connected and completely obstructing the common duct for over 4 hours.

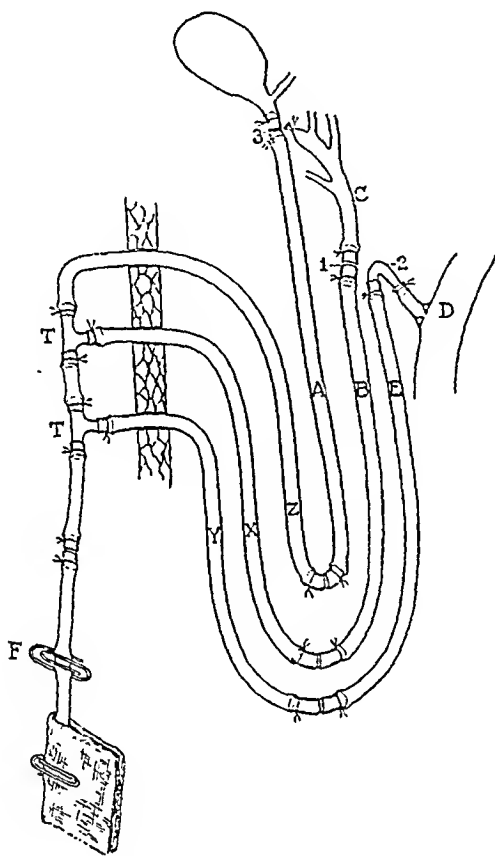
From the failure of the bile column to rise far it was obvious that the gall bladder must be storing the bile almost as fast as it was formed. To test its ability in this connection, 3 cc. of 0.9 per cent sodium chloride solution was run



TEXT-FIG. 2. The pressure developing during the first few hours of total biliary obstruction in an animal with the gall bladder connections left undisturbed. Contrast with Text-fig. 1. The manometer was connected with the common duct for over 4 hours.

abruptly into the common duct through the tube connected with the manometer. The momentary increase in pressure caused by this disappeared almost at once. 5 minutes later 5 cc. more of the solution, injected into the common duct and toward the gall bladder, caused an increase in pressure sufficient to raise the bile column to 300 mm. in the manometer, that is to say, to almost the maximum pressure of total obstruction whether the gall bladder be in or out. There followed a rapid return of pressure to the previous level. Although the manometer remained in connection with the common duct for another hour there was still

In the first 10 minutes but 1.2 cc. of relatively light-colored limpid bile was obtained, though one not nearly so light as liver bile is in an animal which had not fasted. It contained 0.77 mg. of bilirubin per cc. The dog was then fed and in the next 10 minutes 10.2 cc. of a dark, viscid bile having a bilirubin content of 1.61 mg. per cc. flowed into the graduate.



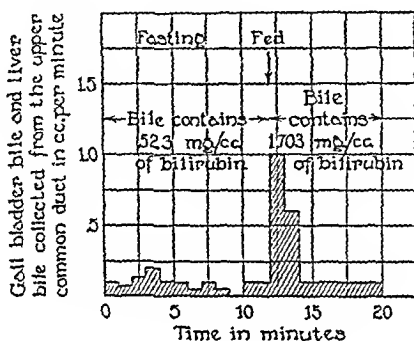
TEXT-FIG. 4. Plan of the "triple intubation." Besides the tubes AB and YE leading from and to the common duct which constitute the so called "altercursive intubation" of previous papers, there is a third tube ZA communicating with the gall bladder. When the tube was clamped at F bile flow to the intestine went on as usual for weeks or months. There was merely a detour of the fluid to the outside.

Proof that the Gall Bladder Expels Bile by Forceful Contraction.

The phenomena as described suggested a forceful discharge of bile from the gall bladder. In order to study them to advantage we

Specimen Protocols.

Dog 9, Text-fig. 3, weight $8\frac{1}{2}$ kilos. 53 days prior to this experiment an "altercursive intubation" was carried out under ether. The gall bladder connection with the common duct was left undisturbed. During the long preliminary period the animal remained in excellent health, and bile was allowed to flow into the intestines as usual, by way of the altercursive detour. The dog was now fasted for 72 hours, after which the tube,—which drained not only the liver but the gall bladder,—was disconnected, and the amount of bile collected from it in each minute was recorded for half an hour. Food was then given



TEXT-FIG. 3. Food as a stimulus to the discharge of bile from the gall bladder. Bile was collected from the upper common duct draining both liver and gall bladder. The gall bladder connections had been left undisturbed. When food was given a large amount of viscid and highly pigmented bile was voided practically at once. Compare the finding with the gradually increasing output of bile from animals with the organ segregated (lower portions of Text-figs. 5 and 6). The differences in the amount and nature of the bile collected point to a discharge of bile from the gall bladder.

while the collection of bile was continued. As the chart indicates, only 1.1 cc. of bile had appeared during the 15 minutes before this. It was light in color, limpid, and contained 0.523 mg. of bilirubin per cc. Pigment determinations on it were carried out by a method described elsewhere.²¹ Within 2 minutes after food was first taken a gush of dark viscid bile appeared (1.6 cc.) which contained upon analysis 1.703 mg. of pigment per cc.

Dog 11, weight $9\frac{1}{4}$ kilos. 18 days before this experiment an "altercursive intubation" had been done under ether. The gall bladder connection had been left undisturbed. Thereafter bile was allowed to flow to the intestines as usual. For the 72 hours previous to the experiment the animal was fasted, after which the bile from the tube draining liver and gall bladder was collected into a sterile graduate and the amounts recorded every 5 minutes.

gall bladder, the resistance to the passage of bile into the intestine, and the amount of liver bile secreted.

With five dogs triply intubated as described we have repeatedly observed upon the taking of food abrupt pressure increases in the column of bile in a manometer connected with the gall bladder,—clear evidence that the organ contracts. The data of one such instance are recorded in Text-fig. 5.

Dog 15, weight 11 kilos, Text-fig. 5. 10 days previous to the experiment a "triple intubation" had been done, with insertion of the third cannula directly in the cystic duct. The animal remained healthy and active. After a fast for 48 hours just prior to the experiment a manometer was connected to the tube leading to the gall bladder, while the tube collecting liver bile was allowed to drain into a sterile graduate. In the text-figure the amount of liver bile secreted in 5 minute periods is plotted in cross-hatched columns. During the first 20 minutes less than 0.5 cc. was obtained in each period and the column of bile in the manometer connected with the gall bladder remained constantly at the 100 mm. level, showing slight fluctuations referable to the respiratory movements.

Food was then offered to the animal and it was allowed to eat for 2½ minutes, consuming in this time about 150 gm. Almost at once the bile column in the manometer, registering pressure changes within the gall bladder, rose to over 200 mm. and it returned again slowly toward its previous level in the course of the next 8 minutes reaching 115 mm. and there remaining. In the meantime food was removed. The column of bile in the manometer remained level until, 17 minutes later, food was again offered. Soon after eating the bile rose above the 250 mm. level, overflowed the manometer, and the experiment was discontinued. Through all this the animal stood quietly making no movements of its body. There is to be noted in the chart a gradual increase in the amount of liver bile secreted after the taking of food. Its flow was constant and continuous. No gush of fluid occurred from the cannula yielding liver bile, such as would suggest a generalized increase in the intraabdominal pressure, and of course no bile could reach the gall bladder from the liver because the tube leading to the viscus had been attached to the manometer. 4 days later while the animal was in excellent condition, it was killed with chloroform. At autopsy all the bile ducts were found normal in appearance and the cannulas patent.

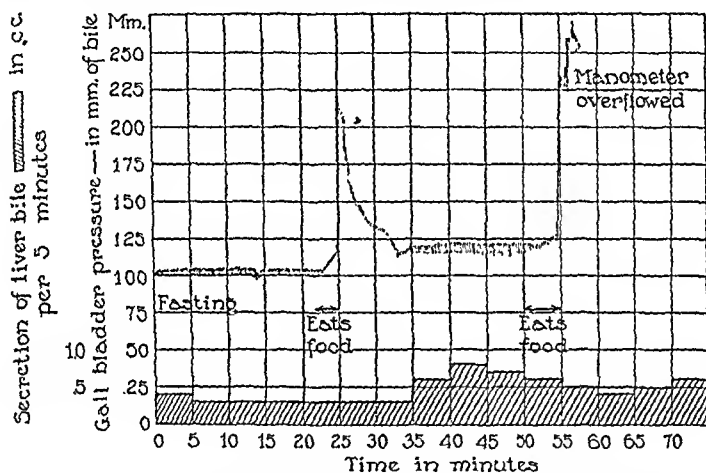
The following instance shows the same phenomenon and its rhythmic recurrence in the absence of any further ingestion of food.

Dog 16, weight 9 kilos, Text-fig. 6. Under ether anesthesia a "triple intubation" was performed, and the three tubes inserted precisely as represented in

resorted to an accessory intubation, whereby a tube was connected directly with the organ in addition to the usual "altercursive intubation." This "triple intubation" already referred to is represented schematically in Text-fig. 4.

The third cannula, 3, connected to the rubber tubes *A* and *Z* was placed either directly in the cystic duct or (as shown in the figure) just far enough below it to allow the tiny duct draining part of the left central lobe of the liver to enter above the point of intubation and thus supply bile directly to the gall bladder. The findings were similar by both methods of intubation.

The ends of the three tubes introduced into the abdomen were passed through



TEXT-FIG. 5. Pressure changes within the gall bladder after the taking of food. The upper curve shows the sudden increases in gall bladder pressure, immediately after eating. The lower columns show the amount of bile secreted by the liver in 5 minute periods.

separate orifices in the lateral body wall and joined outside by means of glass *T*-tubes and short rubber connections. All of these joints were protected with wrappings of phenolized gauze. When a clamp was set upon the drainage tube *F*, bile secreted by the liver into tubes *BX*, upon arriving outside the body, was free to flow in two directions—on into tubes *YE* and thence into the duodenum, or backward into tubes *ZA*, and thence to the gall bladder. This latter course would be taken when the physiological resistance to the passage of bile into the intestine was high. By disconnecting the tubes leading to the gall bladder and to the lower common duct and bringing them into connection with manometers it was possible to measure simultaneously the pressure conditions within the

was now opened for a few seconds during which 3 cc. of bile flowed from it. The pressure fell to 100 mm. but soon rose again to 220 mm. and 5 minutes later to 260 mm. The dog was very quiet during the entire period of the observations.

The 3 cc. of bile collected was dark and viscid and contained 1.47 mg. of bilirubin per cc., this despite the fact that there was an admixture with liver bile derived from the left central lobe of the liver; the 7.3 cc. of liver bile from the remainder of the liver obtained during the 35 minute interval between the taking of food and the collection of bile from the gall bladder was light and limpid and contained but 0.55 mg. of the pigment per cc.

The cross-hatched columns in the text-figure show that there was a slightly increased output of bile by the liver after the feeding.

It is noteworthy that in these two experiments, and in all similar ones as well, there was no sudden gush of bile from the liver, the increase in bile secretion after feeding being very gradual. The abrupt rises in the column of bile in the manometer connected with the gall bladder can therefore have been due only to pressure changes within the viscus. Are they the result of true gall bladder contractions or of extraneous pressure factors?

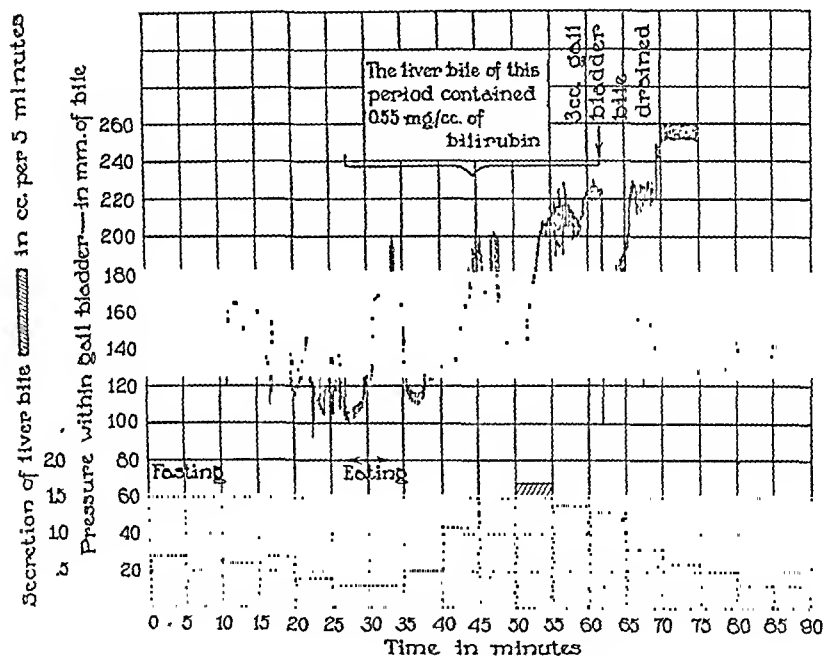
The observed phenomena cannot be the effect of a generalized increase in abdominal pressure consequent upon an increase in the gastric contents; for it is well known²⁶ that this pressure remains constant during the taking of food unless enormous quantities are eaten. Kelling²⁷ has shown that in the case of the dog more than 300 gm. of food can be taken without increasing the intraabdominal pressure, owing to a compensating relaxation of the muscles of the abdominal wall. In some of our experiments (as that shown in Text-fig. 5) the column of bile in the tube connected with the gall bladder rose abruptly when the animal had taken not more than 150 gm. of food, into a stomach previously empty. In several experiments, like that charted in Text-fig. 6, the rhythmic recurrence of abrupt increases in pressure within the gall bladder, in the absence of any further ingestion of food, rules out the possibility that a generalized increase in intraabdominal pressure was the direct cause of the phenomenon. Furthermore in several other instances, none of which have been charted, increases in pressure within the gall bladder did not occur until 10 to 15 minutes after food had been

²⁶ Cannon, W. B., *The mechanical factors of digestion*, London, 1911.

²⁷ Kelling, G., *Z. Biol.*, 1903, xlv, 161.

Text-fig. 4. It will be seen that a small duct from the left central lobe of the liver opened into the cystic duct above the point of intubation. Recovery from the operation was prompt and the animal continued active and healthy.

9 days after operation, and following a fast of 48 hours, the tube connecting with the gall bladder was joined to a manometer. The tube collecting liver bile was allowed to drain freely into a sterile graduate and the amount of bile received was recorded at 5 minute intervals for half an hour. A little food was then given, the animal eating about 150 gm. of the bread, milk, and meat mixture in 5 minutes.



TEXT-FIG. 6. Pressure changes within the gall bladder after the taking of food. The upper curve shows four rhythmic increases and decreases in pressure each of 5 minutes duration or more. The cross-hatched columns record the quantities of liver bile voided. Feeding stimulated secretion.

During the 30 minutes prior to the feeding the gall bladder pressure fluctuated as shown in the chart. Shortly after the first ingestion of food the column of bile rose to 200 mm. and gradually sank again to 120 mm. 5 minutes later it began to rise, again attaining the 200 mm. level and again sinking, this time to 130 mm. 25 minutes after the taking of food it showed another increase, this time to 220 mm., which pressure was maintained for 5 minutes. The tube

usual expulsion of bile at the first taking of food did not occur; and only 15 minutes later did bile flow from the open end of the tube. The amount collected during each minute thereafter was recorded, and is shown in the text-figure. It appeared in sudden gushes, each enduring but a few seconds and followed by a slow ooze from the open end of the tube. These ejections persisted intermittently for 35 minutes and then ceased. As the chart shows, no bile was obtained during certain periods. Of these there were four of 1 minute each, one of 2 minutes, and one of 7 minutes. 6.1 cc. of bile in all were expelled in this way.

At autopsy, performed a week later, after the animal had been killed with chloroform while in excellent health, the bile ducts were all found normal in appearance, and no obstruction was noted. There was a small duct secreting bile into the hepatic duct above the point of intubation. It was found to drain about 40 gm. of liver and, from what is known of the rate of bile secretion in the dog, can have provided but very little of the 6.1 cc. of bile. This latter had the general character of "gall bladder" bile, being dark and viscid and with a pigment content of 1.94 mg. of bilirubin per cc.

The intermittent voidings of bile recorded in the chart suggest that individual contractions of the gall bladder took place at intervals throughout a period of half an hour during gastric digestion. As just mentioned a duct from a small proportion of the liver secreted its bile into the hepatic duct above the point of intubation. But the voidings can hardly be ascribed to the influence of the food stimulus to increase the formation of liver bile, a part of which was voided through the tiny duct. For, as shown in Text-fig. 6, the increase in bile formation which occurred after the taking of food by this same animal was slow and gradual, as in all our feeding experiments. Moreover the bile was voided intermittently from the tube leading to the gall bladder, not continuously, and it had the general characteristics of "gall bladder" bile, being dark and viscid.

Reciprocal Activities of the Gall Bladder and the Musculature about the Lower Portion of the Common Duct.

The assumption that the gall bladder contracts and the sphincter of the common duct relaxes at the same time has been much disputed in the past.

Doyon²⁸ was the first to believe that he had shown such an occurrence. He experimented with the anesthetized dog. Rost²⁹ more recently, through ob-

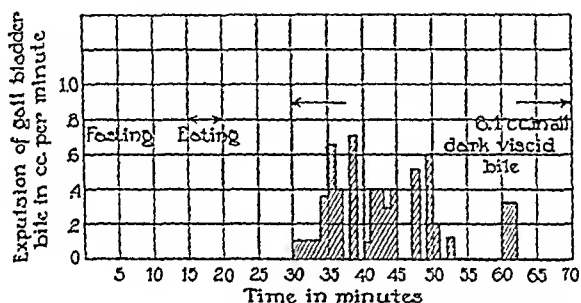
²⁸ Doyon, M., *Arch. physiol. norm. et path.*, 1894, vi, series 5, 19.

²⁹ Rost, F., *Mitt. Grenzgeb. Med. u. Chir.*, 1913, xxvi, 711.

eaten. In an experiment to be described below (Text-fig. 7), the tube leading to the gall bladder was allowed to drain freely into a sterile graduate. Even under these conditions no bile drained from the organ for 10 minutes after food had been eaten. Obviously in this instance and in those cited above increases in the gastric contents had caused no increase in pressure within the gall bladder.

The pressure changes within the viscus endured so long, for example nearly 10 minutes in the case shown in Text-fig. 5, 5 or 10 minutes in that of Text-fig. 6, and occurred at such lengthy intervals, that they cannot be ascribed to the transmission of rhythmic peristaltic pressure changes from the other viscera.

Obviously then, these findings can have come about through

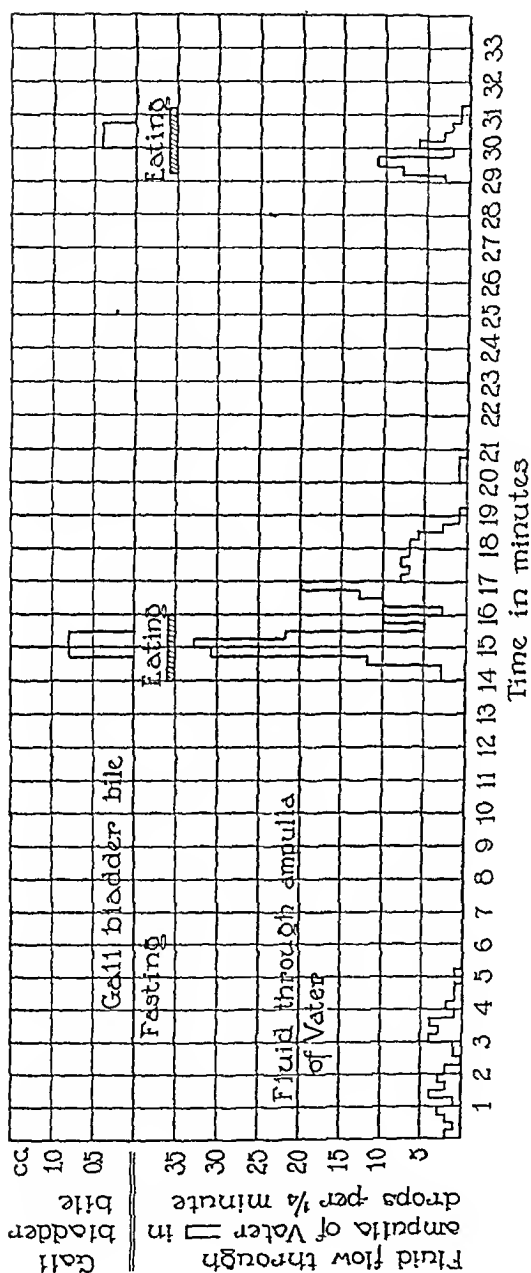


TEXT-FIG. 7. Expulsion of bile from the gall bladder during gastric digestion. The intermittency of the flow is evidence of separate increases in pressure within the organ such as have been charted in Text-fig. 6.

nothing but prolonged forceful contractions of the gall bladder, following the stimulus of taking food, and occurring repeatedly during early digestion. They had the character of rhythmic smooth muscle contractions.

In an accessory experiment in which the same animal was employed, as in the experiment described in the previous protocol, the amount of bile emerging from the intubated gall bladder was studied, not the pressure exerted upon it.

Dog 16, Text-fig. 7. 16 days after a "triple intubation" and following a 24 hour fast the tube leading to the gall bladder was opened, so that it might drain freely into a sterile graduate. For a period of half an hour no bile appeared. 200 gm. of food was then offered to the animal and eaten in 5 minutes. The



TEXT-FIG. 8. Upon feeding there occurs simultaneously an expulsion of bile by the gall bladder and a lessening in the resistance to the passage of bile to the duodenum.

The upper curve shows when bile is expelled from the gall bladder against the resistance offered by a column of bile 200 mm. high, and also the amounts expelled. The lower curve records the periods of decrease in the resistance to the passage of bile into the intestine as evidenced by a flow of fluid to the gut.

servations on the voidings of bile in unanesthetized dogs with the Pavlov biliary fistula,³⁰ came to the same belief. Meltzer³¹ accepted such an occurrence as constituting a special instance of the law of reciprocal innervation.

We have shown in an accompanying paper that at the first perception of the nearness of food and also upon its ingestion the physiological resistance to the passage of bile into the gut relaxes. In the present contribution we have presented evidence that the same stimulus causes a discharge of bile from the gall bladder, presumably by its contraction. In experiments now to be reported we have attempted to determine whether there is a relationship in time between the two phenomena. There is one. They occur synchronously. A type experiment follows.

Dog 19, male, weight 12 kilos, Text-fig. 8. 2 weeks prior to the experiment a "triple intubation," under ether anesthesia, with insertion of the third cannula directly in the cystic duct, was followed by prompt and complete recovery. The dog remained healthy and active, with the bile draining as normally save for the detour.

After a fast of 30 hours the tube connecting with the lower common duct was joined to the "flow" manometer, described in the accompanying paper,¹ for measuring the flow of fluid through the lower common duct at a constant pressure. The bile columns in this manometer were adjusted to exert a pressure equal to that of a 170 mm. column of bile. The flow of fluid was estimated by counting the drops emerging from the nozzle in periods of 15 seconds. The tube connected with the gall bladder was allowed to drain into a sterile, vertical tube of such length that no bile could be collected from it into a sterile graduate at its end until the pressure of a 200 mm. column of bile had been overcome. For the sake of simplicity the amount of liver bile drained by the third tube and collected during the period of the experiment is not recorded in the chart.

During an initial 15 minute period of observation no bile was forced out of the gall bladder and but little passed from the flow manometer into the duodenum.

Food was then given for 2 minutes. Almost at once, 5 to 10 seconds after the first swallow, the bile column began to rise in the vertical tube connected with the gall bladder. In 45 seconds, as shown in the chart, dark viscid "gall bladder" bile was forced into the collecting graduate at the end of this vertical tube, against the 200 mm. of pressure, and it continued to flow for 45 seconds more, delivering 0.6 cc. in all. Then the bile column in the tube fell to the 180 mm. level, so that bile no longer entered the graduate at its end, a finding not shown in the chart. During the next 2 minutes the column fell slowly to the

³⁰ Pavlov, S. P., *Ergebn. Physiol.*, 1902, i, 1. Abt., 246.

³¹ Meltzer, S. S., *Am. J. Med. Sc.*, 1917, clxiii, 469.

We have been able to prove that upon the taking of food some portion of the gall bladder contents is expelled forcibly by contraction of the viscus. But more than this, we have demonstrated the existence of the long supposed and equally as long disputed reciprocal relationship between gall bladder activity and that of the musculature about the lower end of the common duct, as expressed in resistance to bile flow. At the same time that the gall bladder contracts the resistance to the flow of bile into the gut markedly lessens, thus making for a more effective discharge of the gall bladder contents.

The discharge of bile into the duodenum may be thought of as chiefly dependent upon the interaction of three factors, the tonus of the muscles about the lower common duct, the activity of the gall bladder, and the pressure of bile secretion. To make clear the interaction of these in effecting the flow of bile to the intestine let us consider their activities in connection with the taking and digestion of food. The demonstration of the slight differences in pressure causing bile to flow, first toward the intestine, then toward the gall bladder, reveals a truly exquisite mechanism.

In a paper published with this one we have described certain physiological variations in the resistance offered to the passage of bile into the intestine. The "normal" resistance, 4 to 12 hours after a feeding, is sufficient to hold back a column of bile 100 to 120 mm. in height. The resistance increased, during fasting periods often supporting a column of bile 200 to 250 mm. high. Promptly at the mere perception of food and again upon its ingestion a decrease in this resistance was noted, bile flowing through the ampulla of Vater at 50 mm. pressure or even less. The reaction was so immediate that one must suppose it to have been reflex in nature. This decrease in resistance was but transient for the latter soon increased after food had entered the stomach and often became high enough during a brief period to support a 250 mm. column of bile. After a period varying from 10 to 30 minutes the resistance decreased and it fluctuated from high to low as gastric digestion proceeded.

The two other factors which affect the escape of bile into the duodenum—the pressure of bile secretion and the gall bladder activity—have been described in this present paper. We have shown in fasting animals with gall bladder cut off from the duct system that

original level of about 100 mm. Simultaneously with the intake of food, as shown in the chart, the flow to the duodenum occurred indicating a decrease in the resistance thereto and the flow kept up for some time thereafter, its rate indicating a progressive lessening in the resistance, one most pronounced at the precise period when bile was being ejected from the gall bladder. The flow ceased in 5 minutes but 10 minutes later recurred again upon a second brief feeding, as did also the expulsion of bile from the gall bladder. This time the greatest rapidity of flow was not quite synchronous with the expulsion from the gall bladder.

At autopsy 9 days later the bile ducts were found normal in appearance, the cannulas and tubes were open and intact.

In this experiment the pressure within the gall bladder increased sufficiently to force bile out of the organ against the weight of a column of bile 200 mm. in height. No similar expulsion occurred until food was again offered. These pressure increases were synchronous with a decrease in the resistance to the passage of bile to the gut. That only small amounts of bile were obtained from the gall bladder can be accounted for by the fact that it was necessary for the organ to do more work forcing bile out against this artificially high resistance than would be needed to expel the fluid through the normal channels under the condition of lessened resistance prevailing at the time.

In our five dogs with "triple intubation" this experiment was repeatedly carried out, with in each case the same result. Always upon taking food, frequently at the mere perception of it,¹ there was a sudden synchronous decrease in the resistance to the passage of bile into the intestine and an increase in the pressure within the gall bladder as shown by the expulsion of bile. The pressure increase on the part of the gall bladder did not at once reach its maximum, a point shown in Text-figs. 5 and 6, but endured at times several minutes and served to expel several cc. of bile from the viscus, when the resistance thereto was slight (Text-fig. 7).

DISCUSSION.

The observations recorded in this and the accompanying paper would appear to possess a special worth because they were made under controlled circumstances in the healthy, unanesthetized dog.

continues at intervals during the process of gastric digestion. Whether the actual ejection of bile is synchronous with and perhaps dependent upon the gushes of acid chyme through the pylorus cannot be said from our observations. That some relationship does exist is suggested by the fact that the resistance to bile passage into the intestine fluctuates during gastric digestion as further by the intermittent character of the expulsion of bile from the gall bladder.

The contractions of the gall bladder we studied were slow and endured often for several minutes, sometimes coming on quickly, sometimes slowly and wearing off gradually. They were such as to slightly more than double the pressure within the organ. It is of importance to note too that only a little bile was ejected at any one time. This fact may readily account for the failure of previous workers to observe contractions of the gall bladder when they have been sought by methods rendering the organ visible. Of course in such procedures the effects of the operations and the anesthetics employed have militated against success. To explain a supposititious discharge of gall bladder contents in the absence of visible contractions of the organ many authors have invoked the inspiratory increase in pressure on the viscus. Recently, on the basis of new work, Winkelstein¹⁰ and Winkelstein and Aschner^{32,33} have concluded that respiration is the "motor of the gall bladder." During our observations we sometimes noted considerable fluctuations in the pressure within the biliary tract traceable obviously to the respiratory movements. While the dog was breathing or panting quietly the pressure changes were not more than a few mm., scarcely enough to affect the entrance of bile into the gall bladder or its exit therefrom. Usually, the column of bile in the manometer connected with the gall bladder rose and fell about 10 to 15 mm. with each breath. Occasionally, however, on deep inspirations, the pressure often increased 20 to 30 mm. The retching movements preparatory to vomiting raised it to 600 mm., and the straining incident to defecation was once seen to cause a rise in the column of 50 mm. It is to be remembered however that these pressure alterations affected not only the gall bladder but the gut which might

³² Winkelstein, A., and Aschner, P. W., *Am. J. Med. Sc.*, 1924, clxviii, 812.

³³ Winkelstein, A., and Aschner, P. W., *Am. J. Med. Sc.*, 1926, clxxi, 104.

bile is secreted by the liver at a constant rate into a manometer tube until the column of bile has risen in the face of the progressive obstruction to 300 mm., when secretion abruptly ceases. If, however, the gall bladder is still connected with the ducts the pressure of bile developing within the biliary tract as measured by a manometer connected with the common duct remains for some hours below that of a column of bile 175 mm. in height and is usually equivalent to that of a bile column of 100 to 150 mm. When food is eaten there soon occurs within the gall bladder an increase in pressure such as would result from a contraction of smooth muscle, and with it an actual partial emptying of the organ, an activity synchronous with a relaxation of the resistance to the passage of bile to the gut. The phenomenon recurs at intervals during the process of gastric digestion.

It is of importance to note that the pressure developed within the gall bladder in these experiments was amply sufficient to force bile through the ampulla of Vater against the resistance of the muscles in this region, save when the latter were in that temporary state of high tonus, enduring 10 to 30 minutes, which closely follows their immediate relaxation upon the taking of food. It is probable that during this brief period but little bile entered the intestine. Even under these conditions, however, the pressure exerted by the gall bladder contractions about equalled the resistance offered. Later much bile may have entered the intestine, for these contractions, as we have shown, occurred repeatedly during gastric digestion.

From these observations it is possible to correlate the biliary factors which are responsible for the discharge of bile into the duodenum with the taking and digestion of food.

During fasting periods bile is prevented from entering the intestine by the high resistance of the muscles about the lower common duct. The force of secretion is thus directed toward the filling of the gall bladder, which, by its concentrating activity, is able to admit and store large amounts of bile. As result the liver may secrete bile continuously without its appearance at the ampulla of Vater, and the development of a high pressure within the ducts is prevented.

The escape of bile into the intestine occurs promptly at the first ingestion of food, practically ceases then for a short period, and later

dog fasted 24 to 48 hours is usually about equal to a column of bile 100 mm. high. After a few swallows of food there is a rapid increase in the pressure to more than 200 mm. with a gradual fall in it again, and repeated similar rises and falls occur thereafter. The gall bladder contractions responsible for these alterations are accompanied by a lessening in the resistance to the passage of bile to the intestine, a resistance which is maintained by the muscles at the lower end of the common duct. There would appear to be a reciprocal response on the part of the two structures to the one stimulus.

The maximum pressure developing within the temporarily obstructed biliary tract in an animal with the gall bladder excluded about equals that of a column of bile slightly more than 300 mm. in height. The taking of food acts as a stimulus on the rate of bile secretion, but does not alter the maximum secretion pressure. When the gall bladder is connected with the duct system, obstruction does not lead until after some hours to the development of a pressure of more than 100 to 150 mm. within the biliary tract,—that is to say the pressure does not rise above the normal. Its failure to rise further is referable to the activity of the gall bladder to store and concentrate the bile as secreted.

The physiological and clinical significance of these findings is discussed.

receive bile from it and that under these circumstances no flow could occur. As Doyon has pointed out,²⁸ it does not seem that respiratory changes in pressure can be held responsible for the normal discharge of the gall bladder contents. The average respiratory increase in pressure would seem to be insufficient to cause a flow of bile through the ampulla of Vater unless the musculature about the lower common duct is much relaxed as it is just after eating. At such times the mere pressure of bile within the ducts is sufficient for the task, as we have shown in the preceding paper.¹

The facts reported here have an important clinical significance. That the normal resistance to the passage of bile through the ampulla is connected in some way with gall bladder function is sufficiently shown by the absence of this resistance in species lacking the viscus²⁴ and also by the dilatation of the ducts²⁹ and the breaking down of the resistance which occurs in individuals from which the organ has been removed surgically.⁷ Cholecystectomy destroys the mechanism governing the intermittent expulsion of bile that we have described. The fact is of significance for it bears upon the origin of the digestive disturbances occurring in patients after removal of the gall bladder.

A further point deserves mention. The stimulus of food brings about a physiological bile drainage, by relaxation of the muscles about the lower common duct with contraction of the gall bladder. The value of frequent administrations of food to patients when it is desired to promote such drainage is evident. It is conceivable that such feedings will be more efficacious if the food is sufficiently acid to bring about the extreme relaxation of the musculature about the lower common duct noted under experimental conditions when acid is fed.^{1, 35}

SUMMARY.

After feeding a dog, forceful contractions of the gall bladder occur that are sufficient in strength to expel part of the contents of the viscus against a considerable pressure resistance.

The pressure within the gall bladder of a healthy, unanesthetized

²⁴ Mann, F. C., *J. Lab. and Clin. Med.*, 1919-20, v, 107.

²⁵ Cole, W. H., *Am. J. Physiol.*, 1925, lxxii, 39.

over half of the total hemoglobin to exist in the reduced (deoxygenated) state. The "spontaneous deterioration" of hemoglobin to methemoglobin is also an oxidation process and it is influenced by the tension of molecular oxygen in exactly the same way. This gradual deterioration of Hb (so called "spontaneous MetHb formation") which always occurs when blood or Hb solutions are stored under the usual conditions outside the body, can be inhibited or wholly prevented if the hemoglobin is stored in a sealed system in the presence of a biological reducing agent. Thus, when air is excluded, the stability of the active blood pigment is actually increased by the presence of the same biological substances which rapidly oxidize it in the presence of air.

A study has now been undertaken in which it is attempted to apply these principles, as outlined for hemoglobin, to the oxidation and reduction of substances of importance in immunology. It seemed that such a study might well be begun with experiments on bacterial hemotoxins. These substances, which apparently are true antigens, although themselves of relatively little importance, have proved valuable objects of study in the historical establishment of many of the general principles of immunology. The hemotoxin of *Pneumococcus* was the particular one chosen as the subject of the first investigation because the mechanism of the oxidation of this bacterial substance had been established in an earlier paper (3).

The term bacterial *hemotoxin* has been proposed by Přibram (5) to distinguish the bacterial lysins which exhibit antigenic properties from other substances of bacterial origin which although possessing hemolytic properties are not antigenic. It is also important to avoid confusing the hemotoxins with the so called immune hemolysins. The hemotoxins are substances which are specifically (or primarily) toxic for red blood cells, just as diphtheria toxin and tetanus toxin are, respectively, selective for other tissue cells. Again, like the "true" toxins, the hemotoxins are *antigenic*, or more properly speaking "antitoxinogenic," in that when injected into animals they induce the formation of a neutralizing antihemotoxin (analogous to antitoxin). The hemolysins, on the other hand, are "sensitizing" *antibodies* induced by the injection of a primarily non-toxic antigen (red blood cells), and, unlike the hemotoxins, their species-specific hemolytic action is dependent upon the cooperative mechanism of the sensitizing hemolysin and alexin (complement).

Pneumococcus hemotoxin has been described by a number of workers (6, 7) but its first complete study was made by Cole (8) in 1914. It is a true antigen and when injected into animals causes the formation of an antihemotoxin, an antibody which neutralizes the hemotoxin (8). Unlike most bacterial hemotoxins, the hemotoxin of *Pneumococcus* is an endocellular substance liberated into the culture fluid only upon disintegration of the cells. As might be expected, the

STUDIES ON THE OXIDATION AND REDUCTION OF IMMUNOLOGICAL SUBSTANCES.

I. PNEUMOCOCCUS HEMOTOXIN.*

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Medical School, Boston.)*

(Received for publication, December 3, 1925.)

INTRODUCTION.

Certain general principles which were established in previous papers (1, 2) on the biological oxidation-reduction of hemoglobin are reviewed below since they are to be utilized in the present series of studies.

Methemoglobin may be considered as the "inactive" form of hemoglobin, in that it no longer combines with oxygen or carbon monoxide. The essential difference between hemoglobin, the "active" blood pigment, and methemoglobin, its "inactive" oxidation product, is the change of the ferrous iron of the molecule to the ferric state. The conversion of hemoglobin to methemoglobin thus, is a "true" oxidation in the electronic sense (3) and must be distinguished from the process of "oxygenation" involved in the formation of oxyhemoglobin.

The oxidation to methemoglobin may be brought about by biological oxidizing agents, and is a reversible process. Both the oxidation ("inactivation") and the reduction ("reactivation") seem to be induced by the same system; the substances or biological systems which in the presence of molecular oxygen, bring about the oxidation of the hemoglobin, induce the reverse process if air is excluded. Apparently these substances, if not disturbed by the presence of oxygen, are essentially reducing agents, but when oxygen is present, they are changed to oxidizing agents (peroxides or "activated oxygen") which bring about the oxidation of other more difficultly oxidized substances such as hemoglobin. All of the hemoglobin oxidations of this type proceed most rapidly at an oxygen tension of approximately 20 mm., which permits the formation of the oxidizing agents but which also permits

* The major portion of the investigations reported in this and three following papers was carried out in the Department of Bacteriology and Immunology of Harvard University Medical School during the author's tenure of a Traveling Fellowship granted by the General Education Board. I wish to express my appreciation of the courtesies and laboratory facilities extended to me by Dr. Zinsser and his staff throughout the year spent in his department.

and incubated for several hours; after the period allowed for reduction, the bacterial cells were removed by centrifugation, and the bacteria-free supernatant of the mixture was titrated for its hemotoxin content. Controls were included to prove that the bacteria used as reducing agents were devoid of hemolytic activity.

Pneumococcus hemotoxin inactivated by oxidation was obtained by exposing an active pneumococcus extract (Type III) to air for 3 hours at 38°C.

Cultures of *B. coli* and of Anaerobic Bacillus *T* (an anaerobic organism isolated from a wound and morphologically resembling *B. tetani*) were grown in 50 cc. centrifuge tubes under vaseline seal; these cultures were centrifuged and the bacterial cells were suspended in 1.0 cc. of the supernatant broth.

TABLE I.

"Reactivation" of Oxidized Pneumococcus Hemotoxin by the Anaerobic Action of Bacteria.

Amount of pneumococcus extract.	Original (reduced) lysin.	Oxidized lysin.	Oxidized lysin after reduction by <i>B. coli</i> .	Oxidized lysin after reduction by anaerobic bacilli.	Controls on bacterial reducing agents.
cc.					
0.10	++++	—	++++	++++	—
0.05	++++	—	++++	++++	—
0.04	++++	—	++++	++++	—
0.02	++++	—	++++	++++	—
0.01	++++	—	++++	++++	—

The following test mixtures were prepared:

- (1) 0.5 cc. oxidized pneumococcus extract + 0.5 cc. broth.
- (2) 0.5 " " " " + 0.5 " suspension of *B. coli*.
- (3) 0.5 " " " " + 0.5 " " " Anaerobic Bacillus *T*.
- (4) 0.5 " broth + 0.5 cc. suspension of *B. coli*.
- (5) 0.5 " " + 0.5 " " " Anaerobic Bacillus *T*.

These mixtures were sealed with vaseline and incubated at 38°C. for 1½ hours, after which time they were centrifuged at high speed. The hemolytic activity of the supernatants of these mixtures was then titrated. Mixtures (4) and (5), comprising controls on the bacterial reducing agents employed, were always negative whether or not the bacterial cells were removed from the test samples added to the erythrocyte suspensions. The results of the titrations of these mixtures ((4) and (5)) are presented jointly in the protocol under the heading "Controls on bacterial reducing agents."

The protocol of a typical experiment is presented in Table I.

detection of active hemotoxin in filtrates of pneumococcus cultures depends not only upon the liberation of the lysin by cell autolysis, but also upon its protection from air (9). There seems to be no relation between the virulence of the strain of *Pneumococcus* and its hemotoxin-producing capacity. By comparisons of the strains in the collection at the Hospital of The Rockefeller Institute, we have found that the most virulent strains frequently are weak lysin producers, and that avirulent strains as often as not produce larger amounts of the hemotoxin.

It is evident from the foregoing that in pneumococcus hemotoxin we are dealing with a specific antigenic constituent of the bacterial cell.

EXPERIMENTAL.

Methods.—Sterile pneumococcus extracts (10) supplied the hemotoxin. The term "reduced" extract, as used in this paper, refers to pneumococcus extracts which have been protected from oxidation. The term "oxidized" extract denotes extracts in which the hemotoxin has been oxidized by exposure to air in unagitated Erlenmeyer flasks. The pneumococcus extracts employed throughout the present study consisted of the filtered extract of a concentrated suspension of pneumococcus cells which had been disrupted by repeated freezings and thawings. This type of extract (the "complete system" type) (10-13) contains easily oxidized substances which upon exposure to air form oxidizing agents which inactivate the hemotoxin. The differences between this type of pneumococcus extract and the so called "incomplete system" type of extract have already been described (11). The hemolysis tests were made in salt suspensions of well washed sheep or horse erythrocytes. The extracts which were used in most of the experiments were prepared by Dr. Louis A. Julianelle, of the Hospital of The Rockefeller Institute for Medical Research.

"Reactivation" of Oxidized Hemotoxin by the Reducing Activity of Bacteria.

In a preceding paper (14) it was shown that the methemoglobin formed by the oxidizing action of pneumococci or of sterile pneumococcus extracts, could, in the absence of air, be reconverted to hemoglobin by the reducing action of the bacteria. Hence, experiments were made to determine if the same biological reducing agents can reconvert the hemolytically inactive oxidation product of pneumococcus hemotoxin to the original active form. Accordingly, bacteria were added to pneumococcus extracts in which the hemotoxin had previously been inactivated by oxidation. The mixtures were sealed

extracts used as source of hemotoxin were of Type III pneumococci. Since the antihemotoxin is not "type-specific" a heterologous immune serum was used to avoid the specific precipitation of the Type III "S" substance also present in the extracts.)

The results of both of these experiments are given in Table II.

As shown in Table II, the hemolytic substance produced by the anaerobic action of the bacteria upon oxidized pneumococcus hemotoxin possesses heat lability comparable to that of the original reduced hemotoxin. This fact merely suggests that the two are identical. More convincing proof is furnished by the immunological neutralization of the "reactivated" hemotoxin by the antihemotoxin present in pneumo-

TABLE II.

Identity of the Active Hemotoxin in the Original Reduced Extracts and in the Oxidized Extracts "Reactivated" by the Anaerobic Action of Bacteria.

Heat lability.			Neutralization by anti-pneumococcus serum of heterologous type.	
	Hemolysis by 0.01 cc. unheated extract.	Hemolysis by 0.1 cc. extract heated 90 sec. at 55°C.	Hemolysis by 3 hemolytic units previously incubated with 0.005 cc. serum.	
			Normal serum.	Immune serum.
Original active hemotoxin in reduced extract.....	++++	—	++++	—
Hemotoxin inactivated by oxidation and "reactivated" by reduction.....	++++	—	++++	—

coccus immune serum. This antibody is specific and does not neutralize the lytic substances produced by other bacteria.¹

Failure to "Reactivate" Hemotoxin Which Has Been Inactivated by Heat.

Experiments were next made to determine if the inactive product obtained by heating pneumococcus hemotoxin can be "reactivated" by the reducing action of bacterial cells. Obviously, the lysin should be heated as little as possible, else it may be so changed chemically that it cannot be reactivated. It seemed probable from the studies made of heated hemoglobin (1) that "reduced" pneumococcus hemo-

¹ Unpublished experiments to be presented in a subsequent paper.

The results (Table I) of experiments such as that described indicate that the inactive, oxidation product of pneumococcus hemotoxin may be reconverted to an actively hemolytic substance by the action of certain bacteria which are not themselves hemolytic. A sufficient number of control tests have been made to prove that the hemolytic substance produced by the action of these bacterial agents upon oxidized pneumococcus extract is not yielded by the action of the same agents upon the broth medium.

Identity of the Active Hemotoxin in the Original Reduced Extracts and in the Oxidized Extracts "Reactivated" by Reduction.

Experiments were designed to prove that the hemolytic substance yielded by the action of bacterial reducing agents upon oxidized pneumococcus extracts was identical with the original hemotoxin present in the "reduced" pneumococcus extract. These experiments included comparisons of the heat lability of the lysins and tests of their immunological neutralization.

A. Comparison of Heat Lability.—Although bacterial hemotoxins, as a class, are relatively heat-labile, certain degrees of difference in lability exist between the lysins of different bacterial origin, as will be shown in subsequent papers. The hemotoxin of *Pneumococcus* stands out as an exceptionally heat-labile substance. When heated in narrow, thin walled tubes, it is entirely inactivated by 90 seconds exposure to 55°C. This is a considerably greater heat lability than that of most other hemolytic substances. It is conceivable that if the lysin yielded in the preceding experiment differed from the one present in the original reduced extract, it might be found to possess a higher degree of heat resistance than the original pneumococcus hemotoxin.

Comparative heating tests were made on 0.6 cc. of reduced pneumococcus extract and 0.6 cc. of the supernatant of the reduced mixtures of oxidized extract and bacterial cells (mixtures analogous to (2) and (3) in the preceding experiments). The test liquids were placed in narrow thin walled tubes, sealed with vaseline, and heated at 55°C. for 90 seconds in an agitated water bath.

B. Specific Immunological Neutralization.—Tests of the neutralization of the hemolytic substance by the antihemotoxin in pneumococcus immune serum were made as follows: 3 hemolytic units of the original hemotoxin, and the same amount of the hemolytic substance produced by the action of the bacteria upon the oxidized extract, were diluted to 1.5 cc. with salt solution; 0.005 cc. of normal horse serum and 0.005 cc. of the serum of a horse immunized against Type I *Pneumococcus* were added to separate tubes of each of the lysins; after 45 minutes incubation of the mixtures, 0.5 cc. of 20 per cent sheep cells was added to each tube. (The

"Reactivation" of Oxidized Pneumococcus Hemotoxin by Treatment with Sodium Hydrosulfite.

It seemed probable from the preceding experiments, that the "reactivation" of the oxidized hemotoxin by the anaerobic action of bacterial cells represented a reduction process. More definite evidence was obtained by the following tests which are similar to those previously described, with the exception that a chemical reducing agent, sodium hydrosulfite, was substituted for the biological reducing agents previously employed.

Weighed amounts of sodium hydrosulfite ($\text{Na}_2\text{S}_2\text{O}_4$) were placed in large test-tubes. Solutions of the required strength were made in measured amounts of 0.1 M, pH 7.5 phosphate buffer mixtures. The solutions were made up fresh for each test and were used within less than 5 minutes after their preparation.

Preliminary tests were made of the possible hemolytic action of the hydrosulfite itself. If solutions of hydrosulfite were added to broth in sufficient strength to give a concentration of 5 per cent in the reduction mixture, the addition of from 0.02 to 0.10 cc. of the mixtures to 1 cc. of blood cell suspension was not without effect. Upon incubation of the blood cell suspensions containing this amount of hydrosulfite, a part of the blood pigment was converted to methemoglobin. This is in conformity with the reports of Conant and Fieser (15) on the action of the oxidation products of the hydrosulfite. As in the case of the biological reducing agents described in previous papers (1, 2), the hydrosulfite, which essentially is a reducing agent, forms oxidation products in the presence of air which oxidize the blood pigment. This phenomenon is important in tests such as are to be described, from two points of view. First, the "methemoglobinized" red blood cell may be much more difficultly hemolyzed than the unaltered cell. (Reports to this effect in the literature have been partially confirmed by incomplete experiments of our own.) Second, there is also a possibility that the same oxidizing agents which oxidize the hemoglobin in the presence of air, may also oxidize the hemotoxin if the reduction mixture is freely exposed to air *before* its addition to the blood cells.

However, both of these difficulties are readily overcome. Amounts of hydrosulfite too small to injure the blood cell suffice to reduce the oxidized hemotoxin.

toxin heated anaerobically may yield inactive products which cannot be "reactivated" by the reducing agents which serve to "reactivate" the oxidation product of the hemotoxin.

"Reduced" (active) hemotoxin was heated anaerobically for a period just sufficient to destroy its activity. The heating was carried out exactly as described in the preceding experiment. Another portion of the same extract was inactivated by exposure to air. The reduction tests were made by use of mixtures analogous to those described in the experiments reported in Table I. The results are presented in Table III.

From Table III, it is obvious that the biological reducing agents which serve to "reactivate" the oxidation product of pneumococcus

TABLE III.

Attempt to "Reactivate" Heat-Inactivated Hemotoxin.

Amount extract.	Hemotoxin in reduced pneumo- coccus extract.		Hemotoxin in unheated oxidized extract (before action of bacteria).	Hemotoxin in unheated oxidized extract (after action of bacteria).	Hemotoxin in heated oxidized extract (after action of bac- teria).
	Unheated.	Heated.			
cc.					
0.06	++++	—	—	++++	—
0.04	++++	—	—	++++	—
0.03	++++	—	—	++++	—
0.02	++++	—	—	++++	—
0.01	++	—	—	+	—

hemotoxin, fail entirely to "reactivate" the inactive products formed in the anaerobic heating of the reduced hemotoxin. These results serve as a valuable check on the preceding experiments, and indicate, moreover, that the "inactive" products formed in the oxidation of the hemotoxin are quite different from those formed when the hemotoxin is destroyed by heat. Since different products are formed, the reactions involved in the inactivation of the hemotoxin by oxidation and by heat must be of different nature, the inactivation by oxidation apparently being similar in nature to the reversible change of hemoglobin to methemoglobin, while the inactivation by heat is comparable to the destruction of hemoglobin to irreversible blood pigment derivatives.

The results (Table IV) show that the inactive product formed by the oxidation of pneumococcus hemotoxin may be "reactivated" by chemical reducing agents as well as by the reducing action of bacteria. Reducing agents convert the inactive products formed during exposure of the hemotoxin to air, to the original active hemotoxin, but they do not "reactivate" the products formed by the anaerobic heating of the active hemotoxin.

Attempts to "Reactivate" Oxidized Pneumococcus Hemotoxin by the Reducing Action of Sterile Animal Tissues.

In previous studies (12) it has been shown that methemoglobin is formed by action upon hemoglobin by the same agents responsible for the oxidation of the hemotoxin. The oxidized, "inactive" blood pigment can subsequently be converted to "active" hemoglobin by the reducing action of sterile animal tissues *in vitro* (14). It seemed probable that the reducing action of animal tissues would likewise "reactivate" the oxidation product of the hemotoxin. The importance of such an action of the tissues is an obvious one, since the hemotoxin is an antigen of bacterial origin.

A limited number of tests have been made by adding sterile, rabbit testicle to oxidized pneumococcus hemotoxin. In no case was an "active" lysin obtained. The negative results of these experiments, however, do not rule out a reducing action of animal tissue to "reactivate" the oxidized hemotoxin. It is quite possible that the hemotoxin was actually reduced but was combined with the lipoids of the tissues. The literature furnishes many reports of the "inactivation" or neutralization of bacterial hemotoxins by tissues *in vitro*; and the inhibitory action of lipoids upon the hemotoxins has been demonstrated for pneumococcus lysin by Cole (8).

Reduction of Inactive Oxidized Hemotoxin Present in Solutions of the "Protein Fraction" of Pneumococcus Cells.

The hemotoxin of *Pneumococcus*, unlike most bacterial hemotoxins, is endocellular. It is apparently protein in nature, possesses the power of inducing antibody formation, and is destroyed by trypsin digestion (Cole (8)). All this being true, it seemed probable that the

The possible injurious action of the aerobic oxidation products of hydrosulfite upon the hemotoxin can be avoided by adding the samples of the reduced mixtures to the red blood cells with a minimum exposure of the reduced mixture to air. Probably the small samples of the mixtures taken for the hemolysis tests include insufficient hydrosulfite to injure the hemotoxin, or possibly the hemotoxin after combination with the red blood cell is difficult to destroy (in analogy with the relative stability exhibited toward oxidizing agents by hemoglobin after combination with oxygen or carbon monoxide) (2). If sufficiently large amounts of the reduction mixtures are added to the blood cells, enough hydrosulfite is included to reduce the oxyhemoglobin. This, however, does not seem to interfere significantly with the hemolytic titres.

In the experiments tabulated in the protocol to be presented in Table IV the following detailed procedure was used.

0.2 cc. of pneumococcus extract previously inactivated by oxidation, diluted to 1.0 cc. with 0.1 M phosphate solution (pH 7.5), was placed in a series of tubes.

TABLE IV.

"Reactivation" of Unheated Oxidized Pneumococcus Hemotoxin by Reduction with Sodium Hydrosulfite.

	Hemotoxin titrations.		
	0.03 cc.	0.02 cc.	0.01 cc.
Oxidized extract untreated.....	—	—	—
Oxidized extract treated with $\text{Na}_2\text{S}_2\text{O}_4$	++++	++++	++
Heated extract treated with $\text{Na}_2\text{S}_2\text{O}_4$	—	—	—

Another series of tubes containing 0.2 cc. of extract inactivated by heat and similarly diluted, and a third series containing broth instead of pneumococcus extract were prepared.

To these tubes was added 0.2 cc. of various dilutions of sodium hydrosulfite dissolved in 0.1 M phosphate solution (pH 7.5). These mixtures were sealed with vaseline. After allowing time for reduction, varying from 5 minutes in the case of the higher concentrations of the reducing agent to 30 minutes in the case of the lower concentrations, the hemolytic activity of the reduced mixtures was titrated. The samples were added to the red blood cells immediately after the removal of the seal. The reduction, of course, is dependent upon the pH, the temperature, and the time, as well as the concentration of hydrosulfite. The pH was maintained at 7.5, the temperature was approximately 25°C., and the time allowed for reduction was never over 30 minutes. The concentrations of $\text{Na}_2\text{S}_2\text{O}_4$ in the reduction mixtures reported in the table ranged from 1.0 to 0.008 per cent.

The hemolytic test mixtures were incubated for 1 hour at 38°C. The test mixtures were then centrifuged and the hemolytic titres recorded.

The results are given in Table IV.

lowing experiment which would be difficult to attempt with "exocellular" bacterial lysins set free during the stage of active growth of the culture.

The object of the experiment was to determine whether both the oxidation and reduction processes can be induced by bacterial cells of the sort from which the hemotoxin was originally derived. Pneumococci possess both oxidizing and reducing powers (1, 10, 13), the reaction induced being dependent upon the presence or absence of air. Hence, pneumococci can be employed as the agents for both oxidation and reduction processes, by admitting or excluding air. If only young, unautolyzed cells are used, it is possible to remove the bacteria by centrifugation and obtain a supernatant solution which includes no hemolytic substances introduced by the microorganisms used as oxidizing and reducing agents. This point was carefully controlled by parallel tests carried out with mixtures of broth and pneumococci at the time of each reduction and oxidation treatment.

The experiment consisted in several successive reversible oxidations and reductions of the hemotoxin by young, unautolyzed pneumococci. The oxidation in each instance was effected by aeration of the mixture of hemotoxin solution and bacterial cells. After allowing time for the oxidation of the hemotoxin, the bacteria were removed by centrifugation, and the supernatant solution was "titrated" for active hemotoxin. The oxidized solution was then again reduced by adding a fresh suspension of young pneumococci and sealing the reduction mixture from air. When sufficient time had elapsed for reduction, the mixture was centrifuged again to remove the bacteria, and the supernatant of the reduced solution was titrated for active hemotoxin content.

This procedure was repeated with the same original solution of hemotoxin for three successive series of alternate oxidations and reductions. The sequence of conversions of the same solution of hemotoxin from the active reduced form to the inactive oxidized form, and back again, is outlined in Table V.

Solution I represents the original active hemotoxin present in the reduced extract at the beginning of the experiment. The first oxidation of the hemotoxin was effected by the oxidizing agents formed in the extract itself when exposed to air (4). The oxidized extract, owing to the inactivation of the labile cellular components of its oxidation-reduction systems, is no longer able to oxidize hemoglobin or reduce methylene blue and methemoglobin (11). Since the extract itself is rendered devoid of oxidizing and reducing activity after the first oxidation treatment, Solutions II, IV, and VI can be regarded simply as solutions of oxidized hemotoxin. Thus, the successive oxidations (inactivations) and reductions (re-activations) in Solutions II to VI can be referred directly to the oxidizing or reducing action of the young pneumococci added to the respective mixtures.

hemotoxin might be included in the "protein" fraction of the *Pneumococcus* which is precipitated by acetic acid in the cold (16). A solution of the "pneumococcus protein" furnished by Dr. Avery was used in the following experiment.

This solution had been prepared from Strain F, a Type IV *Pneumococcus*, by the method described by Avery and Heidelberger (16). It contained 2.8 mg. of protein in 1 cc., had not been protected from air, and was several weeks old when used.

Preliminary tests showed that the solution was non-hemolytic when an amount as large as 0.3 cc. was added to 0.5 cc. of red blood cells. This indicated that if the hemotoxin had been precipitated by the acid, it was present in the inactive form.

Reduction tests similar to those described in experiments with the oxidized "pneumococcus extracts" were made. Two portions of 0.5 cc. of the protein solution were placed in separate tubes; to one of the tubes, 0.5 cc. of 0.1M phosphate solution was added; 0.5 cc. of 0.4 per cent solution of hydrosulite was added to the other tube. Both tubes were sealed. At the end of the 15 minute period which was allowed for reduction, portions of the "reduced" solution and of the phosphate-diluted control were added to 0.5 cc. of red blood cells. The volume of each hemolysis test mixture was adjusted to 1.0 cc. by the addition of salt solution. After 1 hour's incubation at 38°C., the tests were centrifuged and observations made of the hemolysis.

Samples as large as 0.6 cc. of the phosphate-diluted control gave no hemolysis, which confirmed the previous observation of the absence of active lysin in the protein solution. On the other hand, in the case of the solution treated with the reducing agent, samples as small as 0.03 cc. gave complete hemolysis.

The results of this experiment indicate that the *pneumococcus* hemotoxin is precipitated by acetic acid in the cold, and is included in solutions of the "protein fraction" of the cell. Although the hemotoxin is not oxidized rapidly in solutions of washed cells in the absence of other easily oxidized substances (4), it is probably slowly oxidized during the preparation and storage of the protein solutions. There is every reason to believe that the inactive lysin which was "reactivated" in the above experiment is identical with the reversible oxidation product of *pneumococcus* hemotoxin which has been reported in the preceding experiments with sterile *pneumococcus* extracts.

Reversibility of the Oxidation-Reduction of Pneumococcus Hemotoxin.

The endocellular nature of *pneumococcus* hemotoxin, which is not liberated into the culture fluid until disruption of the cell membrane, made possible the fol-

Control Tests with Mixtures of Broth and Pneumococci.—Broth was treated in exactly the same manner as the solutions of the hemotoxin. Upon removal of the bacteria by centrifugation after each oxidation and reduction period, the supernatant of these mixtures was tested for hemolytic activity. They never contained a trace of hemolytic substances, a fact which can be taken as proof that no hemotoxin was liberated by the cells used in the experiment proper.

The results of this experiment (Table V) illustrate the reversible oxidation-reduction of pneumococcus hemotoxin by pneumococcus cells. This experiment is analogous to one upon the reversible oxidation-reduction of blood pigment by pneumococci (Table II, in a previous paper (14)). Here, too, longer periods of time were required for the anaerobic reduction of the hemotoxin than for its oxidation in the presence of air.

COMMENT.

Pneumococcus hemotoxin is an actual constituent of the bacterial cell. In the reduced condition it possesses certain properties which are lost upon oxidation. As it exists in the cell it is protected by the reducing activity of the living bacteria. If the cells are sufficiently exposed to air, oxidizing agents are formed which oxidize the hemotoxin. If, however, the exposure to air is not too prolonged, nor sufficiently drastic to inactivate completely the reducing activity of the cells, this cellular constituent can be reconverted to the original reduced substance whenever the tension of molecular oxygen tension does not mask the native reducing powers of the bacterial cells. The failure of the reduction systems present in the sterile filtered extracts to "reactivate" subsequently the oxidized hemotoxin in aerated extracts is analogous to the loss of their ability to reduce methylene blue and methemoglobin. As previously shown, the loss in reducing power is due to the inactivation of the thermolabile constituent of the oxidation-reduction system (11). Apparently, these thermolabile constituents are more readily inactivated or "overtaxed" in the sterile, filtered, cell extracts than in the living intact bacterial cell.

While the hemotoxin itself may be of no physiological importance, it is highly probable that the reversible oxidation-reduction of some other thermolabile cellular constituents is involved in the maintenance of the physiological activity of bacterial cells.

TABLE V.
The Reversible Oxidation and Reduction of Pneumococcus Hemolysin by Living Pneumococcus Cells.

Oxidation or reduction mixture.	Treatment.	Oxidizing or reducing agent.	Period of oxidation or reduction.	Hemolytic activity.					
				Amounts of mixture expressed in terms of original extract.					
				0.06 cc.	0.03 cc.	0.02 cc.	0.01 cc.	0.006 cc.	
Solution I	Original solution.	Sterile pneumococcus extract scaled from air.		++++	++++	++++	++++	++++	+++
Solution II	Solution I after oxidation.	Sterile pneumococcus extract in the presence of air.	1.5 hrs.	—	—	—	—	—	—
Solution III	Solution II after reduction.	Young, intact pneumococci in the absence of air.	2.5 "	++++	++++	++++	+	—	—
Solution IV	Solution III after oxidation.	Young, intact pneumococci in the presence of air.	1.0 hr.	—	—	—	—	—	—
Solution V	Solution IV after reduction.	Young, intact pneumococci in the absence of air.	3.0 hrs.	++++	++++	++	±	—	—
Solution VI	Solution V after oxidation.	Young, intact pneumococci in the presence of air.	0.5 hr.	—	—	—	—	—	—
Solution VII	Solution VI after reduction.	$\text{Na}_2\text{S}_2\text{O}_4$ in the absence of air.	10 min.	++++	++++	++++	++	+	+
Supernatant of controls (broth plus young pneumococci) for each reduction and oxidation mixture.				—	—	—	—	—	—

SUMMARY.

Pneumococcus hemotoxin, an antigenic substance of bacterial origin, is converted by oxidation to a product devoid of hemolytic action. The oxidation product of the hemotoxin may be converted to the original hemolytic substance by reduction, by the anaerobic action of certain bacteria, or by sodium hydrosulfite. The active lysin, or hemotoxin, produced by the reduction of the inactive oxidized extracts is identical with the original, active, reduced hemotoxin; it possesses the same degree of thermolability and is neutralized by the same specific antibody. The inactive products formed by heating the hemotoxin anaerobically cannot be "reactivated" by reducing agents. The immunological significance of these relations will be discussed in a subsequent paper.

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attributable to lipoidal serum constituents. A wide variation may occur in the inhibitory action of the sera of different, normal rabbits (20).

The hemotoxin of the Welch bacillus is reported to be a true antigen, inciting an immunity response when injected into animals. The production of a specific antihemotoxin neutralizing the hemotoxin has been reported by Schlossberger (21), Wuth (22), and others. The specificity is apparently a "species specificity;" the hemotoxin is not neutralized by sera produced by immunization with other anaerobic bacteria such as the gas edema bacillus (22), nor does the Welch antihemotoxin neutralize the hemotoxin of the tetanus bacillus. Studies on the neutralization of the hemotoxin by the immune antihemotoxin have been made by Henry (9).

EXPERIMENTAL.

Methods.

The methods used in the following experiments are essentially the same as those employed in the previous study (1). Well washed sheep cells were used in the hemolysis tests. Approximately constant concentrations of cells were obtained by colorimetric standardization of the stock suspensions from which the dilutions were made.

The three strains of the Welch bacillus used in the experiments were isolated from heated stools by Dr. Robert Nye of the Boston City Hospital. The cultures were grown in broth, previously boiled and sealed with a heavy layer of vaseline. Infusion broth yielded more active fluids than did meat extract broth. The culture fluids used as the source of the hemotoxin were not filtered but were rendered free from bacterial cells by prolonged centrifugation.

The Influence of the pH of the Hemolysis Test System upon the Activity of Welch Lysin.

It was necessary first to determine the influence of the pH of the medium in which the blood cells are suspended. For this purpose, comparisons were made of the activity of the lysin upon cells suspended in physiological salt, with its activity upon cells suspended in 0.1 M phosphate solutions ranging from pH 7.0 to 8.0. The following tests were made: (1) "titrations" of the smallest amount of lysin required for the hemolysis of cells suspended in the different solutions; (2) comparisons of the time required for hemolysis of a constant volume of cells by a constant amount of lysin; (3) comparisons of the degree of hemolysis effected by a constant amount of lysin in a constant time. Protocols illustrating the results of these experiments are given in Table I.

It is evident (Table I) that the Welch lysin is much less active when the blood cells are suspended in phosphate solutions of pH 7.5 to 8.0, than if the cells are suspended in salt solution or in pH 7.0 phosphate.

STUDIES ON THE OXIDATION AND REDUCTION OF IMMUNOLOGICAL SUBSTANCES.

II. THE HEMOTOXIN OF THE WELCH BACILLUS.

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INTRODUCTION.

The preceding paper reported a study of the oxidation-reduction of pneumococcus hemotoxin. Another hemotoxin, the lysin of the Welch bacillus, has been used as the subject of the present study. In contrast to the pneumococcus hemotoxin, which is endocellular and an actual constituent of the bacterial cell, the Welch lysin is an "exocellular" substance which is liberated into the culture fluid prior to cell disintegration.

The early work of Welch and Nuttall (2), Welch and Flexner (3), and Herter (4) established the existence and suggested the importance of the Welch bacillus. The studies of Bull (5) and of Bull and Pritchett (6) proved the existence of a true toxin in cultures of this organism. While the present paper is concerned only with the hemolytic substance, the hemotoxin, it is desirable to differentiate this lysin from the mixture of other toxic substances which may be contained in culture fluids of the Welch bacillus. Kojima (7) reports three toxic substances: the hemotoxin, a "false" toxin, and a true toxin. The "false" toxin and the true toxin seemed to be quite distinct, the hemotoxin and the true killing toxin were less sharply differentiated, although the hemotoxin proved to be somewhat more thermolabile. Henry (8, 9) distinguishes between the hemotoxin and the true killing toxin which he terms "myotoxin" and attempted to separate the two substances by selective absorption.

The occurrence of the lysin and its properties have been studied by a number of workers (4, 9, 19). Most authorities agree that it is thermolabile; it is non-dialyzable and may be precipitated by alcohol, ammonium sulfate, and other protein precipitants. It is inhibited or neutralized to some extent by normal serum from different animals (16, 20), at least a part of this action probably being

ences at different pH. To show that this is not the case, a comparison of Welch lysin and tetanolysin is also presented in Table I. It is seen there that, while affected to some extent by the pH of the medium in which the cells are suspended, tetanolysin does not show the marked differences in activity which are exhibited by Welch lysin. This also seems to rule out any difference which might be due to slight differences in tonicity of the buffer mixtures at different pH or to effects due to shifts in the ratio of potassium and sodium in the different buffer mixtures.

This influence of pH upon the activity of Welch lysin is of importance in two other connections. First, the pH of the circulating blood is not the optimum reaction for hemolysis by Welch lysin and this in itself, together with the inhibitory action of normal serum, offers a certain degree of protection against *in vivo* hemolysis. Second, many of the early reports that "neutralized" Welch culture fluids were devoid of lytic action may be explained by the fact that fluids "neutralized" to phenolphthalein (above pH 8.5) are too alkaline to offer a fair test for the activity of Welch lysin.

Heat Lability of Welch Lysin.

In most of the experiments on the heat lability of Welch lysin the fluids heated were at pH 6.5; the heating tests were made in vaseline-sealed tubes. It was found that the lysin is destroyed rapidly at 55°C., since 10 minutes at this temperature suffices for its complete destruction. These results indicated a somewhat higher degree of lability than that reported by the majority of the previous workers (4, 9, 11, 14, 16). In view of the effect of pH upon heat inactivation, further experiments were made with the lysins of three strains adjusted to pH ranging from 3.0 to 9.0. All of these were inactivated by 10 minutes exposure to 60°C. (no tests were made at 55°C.).

Inactivation of Welch Hemotoxin by Exposure to Air and "Reactivation" of the Oxidized Lysin by Treatment with Reducing Agents.

A number of preliminary experiments showed that actively hemolytic fluids of Welch bacillus cultures lost their hemolytic power after exposure to air in Erlenmeyer flasks at room temperature. Since the hemotoxin proved perfectly stable for months in sealed tubes, it seemed probable that the inactivation process was an oxidation.

It now follows that comparisons of the lysin content of culture fluids of different pH will be valid only if precautions are taken to maintain a constant pH in the hemolysis test systems. Hence, in all of the following experiments, the blood cells were suspended in buffer mixtures and control tests were made to prove that the acidity or alkalinity

TABLE I.

Comparative Influence of Phosphate Mixtures of Different pH upon the Activities of Welch Lysin and Tetanolyisin.

(a) "Titration" of the lysins.

Welch lysin.					Tetanolyisin.				
Amount of lysin.	Hemolysis of blood cells suspended in				Amount of lysin.	Hemolysis of blood cells suspended in			
	NaCl	pH 7.0 phosphate.	pH 7.5 phosphate.	pH 8.0 phosphate.		NaCl	pH 7.0 phosphate.	pH 7.5 phosphate.	pH 8.0 phosphate.
cc.					cc.				
0.03	++++	++++	++++	-	0.009	++++	++++	++++	++++
0.02	+++	++++	++	-	0.006	++++	++++	++++	+++
0.01	+	+++	-	-	0.003	++	+++	++	++

(b) Comparison of relative degree of hemolysis by a constant amount of lysin in 1 hr. at 38°C.

Blood cells suspended in	Relative degree of hemolysis.	
	Welch lysin.	Tetanolyisin.
	per cent*	per cent*
NaCl	90-95	70-75
pH 7.0 PO ₄	100	100
pH 7.5 PO ₄	20	90-95
pH 8.0 PO ₄	Less than 10.	75-80

* The per cent hemolysis presented in the table is relative and is calculated by considering hemolysis in the pH 7.0 phosphate as 100 per cent.

ity of the fluid added in the lysin tests was compensated by the buffered suspension medium.

If the differences in the hemolytic activity of Welch lysin in the different solutions in which the blood cells were suspended were due simply to some effect which rendered the blood cells more "fragile," one would expect other bacterial lysins to show the same marked differ-

Comparison of the Heat Lability of Reduced (Active) Welch Lysin with the Lability of the Oxidized (Inactive) Lysin.

The following experiment compares the degree of heat resistance possessed by the oxidized and the reduced lysin. The heating treatments were chosen from the data of preliminary experiments, so that one series was heated a period (10 minutes at 55°C.) just sufficient to destroy completely the reduced hemotoxin, and the other series was heated a period (2½ minutes at 55°C.) which had been found sufficient to inactivate somewhat over half of the reduced hemotoxin. Both the reduced and the oxidized lysin were heated in vaseline-sealed tubes. The protocol of this experiment is given in Table III.

TABLE III.

Comparison of the Heat Lability of Reduced and Oxidized Welch Lysin.

Amount of lysin.	Unheated.			Heated 2½ min. at 55°C.			Heated 10 min. at 55°C.		
	Reduced lysin.	Oxidized lysin.		Reduced lysin.	Oxidized lysin.		Reduced lysin.	Oxidized lysin.	
		Not treated with reducing agents.	After treatment with hydro-sulfite.		Not treated with reducing agents.	After treatment with hydro-sulfite.		Not treated with reducing agents.	After treatment with hydro-sulfite.
cc.									
0.2	++++	—	++++	++++	—	++++	—	—	—
0.1	++++	—	++++	++++	—	+++	—	—	—
0.05	++++	—	++++	+	—	±	—	—	—
0.02	++++	—	++++	—	—	—	—	—	—
0.01	++++	—	++++	—	—	—	—	—	—

The data presented in Table III show that the oxidized and reduced Welch lysin possess identical heat resistances. Not only are both destroyed entirely by 10 minutes exposure to 55°C., but the rate of destruction of the two substances is apparently the same, since approximately equal amounts of the oxidized and the reduced substance remain undestroyed after 2½ minutes heating. In view of the important change undergone by the lysin molecule when it is oxidized, the retention of its original degree of heat resistance is remarkable.

Influence of pH upon the Oxidation of Welch Lysin.

The following experiment is concerned with the effect of pH upon the subsequent stability of hemotoxin previously formed in broth of

Tests were next made to determine if the inactive product of the Welch lysin, like the inactive oxidation product of *pneumococcus* hemotoxin, can be converted to the original, actively hemolytic substance by the action of reducing agents. These experiments were performed in the manner described in the preceding paper.

The protocol of a typical experiment is given in Table II.

The results given in Table II prove that Welch lysin which has been inactivated by aeration can be "reactivated" by the reducing action of bacteria or of sodium hydrosulfite. Hence, as previously demonstrated for *pneumococcus* hemotoxin, aeration of Welch bacillus culture fluids yields a reversible oxidation product which can be converted to the originally active substance by reduction treatment.

TABLE II.

"Reactivation" of Oxidized Welch Lysin by Treatment with Reducing Agents.

	Hemolytic activity.		
	Amount of culture fluid.		
	0.1 cc.	0.03 cc.	0.01 cc.
Oxidized lysin (untreated).....	—	—	—
Oxidized lysin treated with hydrosulfite.....	++++	++++	++++
Oxidized lysin treated with non-hemolytic anaerobic bacilli.....	++++	++++	++++

Attempts to "Reactivate" Welch Lysin Which Has Been Inactivated by Heat.

Experiments analogous to those described in the preceding paper were also made to determine if heat-inactivated Welch lysin can be "reactivated" by reduction. The results of these experiments were the same as those with *pneumococcus* hemotoxin; heat-inactivated Welch lysin cannot be "reactivated" by the action of reducing agents. This fact demonstrates that the products formed when Welch lysin is inactivated by heat are distinctly different from the inactive products formed when the lysin is exposed to air.

TABLE IV.
Influence of pH upon the Oxidation of Welch Lysin.

Hemolytic activity of centrifuged culture fluid adjusted to different pH, after aeration.												
Amount of fluid.	Original lysin, before exposure to air.	Lysin adjusted to pH 8.8; after exposure to air for			Unadjusted lysin (pH 7.2)*; after exposure to air for		Lysin adjusted to pH 6.0; after exposure to air for		Lysin adjusted to pH 5.5; after exposure to air for		Lysin adjusted to pH 4.5; after exposure to air for	
		4 hrs.	9 hrs.	24 hrs.	9 hrs.	24 hrs.	9 hrs.	24 hrs.	9 hrs.	24 hrs.	9 hrs.	24 hrs.
cc.												
0.20	++	++	+	-	++	++	++	++	++	++	++	++
0.10	++	++	++	-	++	++	++	++	++	++	++	-
0.05	++	++	+	-	++	++	++	++	++	++	++	-
0.04	++	++	-	-	++	++	++	++	++	++	++	-
0.03	++	++	-	-	++	++	++	++	++	++	++	-
0.02	++	++	-	-	++	++	++	++	++	++	++	-
0.01	++	++	-	-	++	++	++	++	++	++	++	-
0.005	++	++	-	-	++	++	++	++	++	++	++	-
0.003	+	+	-	-	+	+	+	+	+	+	+	-

* Original pH 6.5, which increased to pH 7.2 after a short period of exposure to air.

the optimum pH for lysin production. Cultures of the Welch bacillus were grown in poorly buffered infusion broth the initial reaction of which was approximately neutral. Two series of the lysin-containing broth were then adjusted to different pH by the addition of sterile acid and alkali. One series was protected from air by a heavy vaseline seal; the other series was freely exposed to air. At different periods of time, the stability of the hemotoxin in both the sealed and aerated series was compared by titrations of the hemolytic activity.

In the lysin titrations themselves each hemolytic test system was at approximately the same pH. Control tests were made to prove that the addition of each amount of lysin used did not change the pH of 2.5 cc. of 0.1 M phosphate solution (pH 7.0). Only the larger amounts of the more acid and alkaline lysins caused any change in pH; these cases were controlled by substituting pH 6.8 or pH 7.2 phosphate solution in the indicated instances.

The lysin was added directly to 2.5 cc. of 0.1 M phosphate solution (pH 7.0); after mixing the lysin and the buffer solution 1 drop of a 40 per cent suspension of blood cells was added. The hemolytic test mixtures were incubated 1 hour at 38°C. and then centrifuged.

Considerable precipitation occurred in the acidified lysin, especially in the sealed tubes. As far as was possible, this precipitate was avoided and the clear supernatant was taken for the samples used in the hemolytic tests. The pH was determined colorimetrically at the time of the 13 hour tests.

The results of the tests of the series in sealed tubes showed that Welch lysin retains its activity for at least 24 hours within a pH range of 6.0 to 9.0, provided air is excluded. This fact indicates that differences in the rate of inactivation of the lysin in the aerated series during the period of the experiment can be referred to the effect of pH upon oxidation of the lysin. The results of the tests of the aerated series are presented in Table IV.

To avoid the complications introduced by acid precipitation of the lysin, it is advisable to limit the analysis of Table IV to a comparison of the rate of inactivation of the lysin in the fluids adjusted to pH 6.0, 7.2, and 8.8. In the pH 8.8 fluid, most of the lysin was inactivated after only 4 hours exposure to air. All active lysin had disappeared in the pH 7.2 and pH 8.8 fluids in less than 24 hours, although traces of active lysin persisted in all of the more acid fluids for over 48 hours. The lysin proved most stable in the fluid adjusted to about pH 6.0. From these results it is apparent that within the range of pH 6.0 to

in the reduction of the reversible oxidation product of hemoglobin and of pneumococcus hemotoxin. On the other hand, the heating of the hemotoxin yields "inactive" products which cannot be "reactivated" by reduction treatment.

The active reduced form and the inactive oxidized form of the lysin show no difference in the irreversible change brought about by heat. It seems logical to assume that the heat inactivation is in the nature of a protein denaturization. On the other hand, the marked change induced in the lysin by oxidation must be of entirely different nature and similar to those involved in the conversion of hemoglobin to its reversible oxidation product, methemoglobin.

The rate of oxidation of Welch lysin is influenced by the pH of the culture fluid. If the lysin is exposed to air in fluids adjusted to approximately pH 6.0, the oxidation proceeds much more slowly than in systems more alkaline than pH 7.0. In broth adjusted to more acid zones, around pH 4.0 and pH 5.0, the lysin is more stable than when aerated in alkaline broth, but much of the lysin is precipitated in the more acid systems. In considering the influence of pH upon oxidation of the lysin, it must be remembered that the hemotoxin of the Welch bacillus, like the lysin of *Pneumococcus*, may be oxidized, not by the direct action of molecular oxygen, but by oxidizing agents formed during the oxidation of other substances in the culture fluid. If this is the case, the observed influence of pH may be due not to the direct effect of pH upon the ease of oxidation of the lysin itself, but to its effect upon the formation of oxidizing agents from other substances.

It is probable that the gradual deterioration of Welch lysins which occurs in culture fluids stored under usual laboratory conditions is due in large part to oxidation processes. Where the "deterioration" really is an oxidation, the conditions determining the ease of oxidation of the lysin will determine its stability. Among these conditions, pH has been shown to be very important, and it is probable that the pH of the fluids explains the relatively high degree of stability of the Welch lysin in our broth cultures. When stored at room temperature under vaseline seal in broth cultures with a final pH of approximately 6.4, the lysin activity remained practically unchanged for months. (Since the Welch bacillus is not a strongly proteolytic organism, the hemotoxin, which is probably protein in nature, is not apt to be inactivated by

9.0, the rate of oxidation of Welch lysin is at least roughly proportional to the alkalinity. On the other hand, in fluids of lower pH, unless precipitated by excessive acidity, the lysin is relatively stable in contrast with its rapid inactivation when exposed to air in slightly alkaline solutions.

The unadjusted culture fluid becomes more alkaline when exposed to air; and at least a part of the rapid change from approximately pH 6.5 to pH 7.2 can be explained by the loss of carbon dioxide. Since the lysin is oxidized more rapidly in alkaline solutions, the considerable increase in pH which occurs when the culture fluid is exposed to air must accelerate the inactivation of the lysin. In that sense, the processes involved in the inactivation of the Welch hemotoxin in aerated solutions can be considered as autocatalytic.

*Detection of the Presence of Inactive Hemotoxin in Culture Fluids
Containing No "Active" Lysin.*

A number of 2 months old cultures of Welch bacilli were examined in the usual way for the presence of active lysin. The vaseline seals of some of the cultures had been blown off by the vigorous gas formation; others, with intact seals, were in broth more alkaline than pH 7.5. None of these cultures showed the presence of any active lytic substance in tests with as much as 1.0 cc. of the fluid. However, after the bacteria-free fluids were treated with the reducing agent, amounts as small as 0.02 cc. gave complete hemolysis.

These results are easily explained, of course, by the preceding experiments. They show, however, the possibility of demonstrating the presence of oxidized lysin in culture fluids which would be pronounced free of hemotoxin if examined in the ordinary way. It would seem desirable to apply such tests to all culture fluids of unknown origin before pronouncing the tested strain to be devoid of the power of producing an active lysin.

DISCUSSION.

The hemotoxin of the Welch bacillus is inactivated by exposure to air. The inactivation is an oxidation process and is reversible. The reversal can be brought about by biological agents or by the chemical agent, sodium hydrosulfite, the same agents that is to say, which served

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proteolytic hydrolyses.) On the other hand, in culture fluids of final pH 7.5, the lysin gradually deteriorated after the 2nd week's storage at 37°C. and was completely inactive after 3 weeks. This occurred in cultures under a heavy seal of vaseline and under conditions identical in all respects, except that of pH, with those of the cultures at pH 6.2 which retained their original lysin activity. Since the inactive product in these sealed tubes of the pH 7.5 fluids was "reactivated" by treatment with hydrosulfite, it is evident that the observed "deterioration" of the lysin was an oxidation. While traces of oxygen may have passed through the seal, it would seem that the reducing action of the broth itself would be sufficient to compensate for these traces of air. Possibly, in alkaline systems the direct participation of oxygen is not required in the oxidation of the Welch lysin. At any event, it appears that in alkaline systems (as pH 7.5), the "active" reduced lysin is slowly converted to its "inactive" oxidation product even though the system possesses sufficient reducing activity to prevent the conversion of methylene white to methylene blue.

If the Welch lysin be considered as an antigen of bacterial origin the results of the above investigation are of considerable immunological interest from various theoretical points of view. Among the most suggestive relations illustrated are the following: the reversible oxidation and reduction of an antigenic substance; the inability of the oxidation product to injure (hemolyze) the cell; and the marked influence of pH upon the oxidation of the hemotoxin. These relations suggest a number of potential applications to more important antigens, which it is fruitless to discuss until immunological evidence has been accumulated.

SUMMARY.

A study has been made of the oxidation and reduction of the hemotoxin of the Welch bacillus. The oxidation of the lysin results in the loss of its hemolytic activity. The "inactive" oxidation product of the Welch lysin may be reversed to the "active" lysin by treatment with biological or chemical reducing agents. The lysin, both in its reduced and oxidized form, is irreversibly inactivated by the same heat treatment. The pH of the system is an important factor in the oxidation of the lysin, the process proceeding much more rapidly at a pH above 7.0.

Tetanolysin has been chosen for the purposes of this investigation, as an example of an antigen of bacterial origin. This lysin has more than ordinary interest, since it has been used in classical experiments, the results of which have been applied in the establishment of many of the fundamental principles of immunology. The experiments reported here are concerned with the conditions governing the oxidation-reduction of tetanus hemotoxin (tetanolysin), and with the nature of the hemolytically inactive products formed during the oxidation of the active lysin.

EXPERIMENTAL.

Methods.

The methods used in this investigation were essentially the same employed in the preceding studies (1, 2). A typical strain of tetanus bacillus, which was furnished by Dr. Charles Krumwiede of the Bureau of Laboratories of the New York City Department of Health, was used in all of the experiments.

In measuring the lysin content of culture fluids treated with hydrosulfite, it is important to avoid exposure of the "reduction mixture" to air before its addition to the red blood cell. The possibility of the destruction of the lysin by neglect of this factor was pointed out in the first paper (1).

In later experiments, the following procedure has proved satisfactory. The mixtures of lysin fluid plus reducing agent were sealed with vaseline as previously described. After a reduction period of 10 to 15 minutes, measured amounts of the reduced fluids are added immediately to the blood cell solution used in the final hemolysis tests. The blood cells are held in a cold water bath at 8–10°C. for 15 to 20 minutes before being placed at 38°C. The test mixtures are shaken several times during the preliminary incubation period at low temperature. This procedure has proved more satisfactory than incubating them immediately at 38°C. Comparative lysin titrations of unoxidized fluids not treated with hydrosulfite with the same fluids treated with the reducing agent have emphasized the importance both of rapid addition of the test samples to the blood cells, and of the preliminary period at low temperature.

If the hydrosulfite-treated fluids are exposed to air for even a few minutes, some of the lysin is destroyed; if the aeration is prolonged for an hour and a half, practically all of the lysin may be destroyed. This rapid destruction of the lysin is undoubtedly due to agents formed from the hydrosulfite in presence of air, since the mixtures of lysin fluid plus phosphate solution are not rapidly destroyed when exposed to air.

Relations very similar to the above were encountered by Conant and Fieser (25) in the determination of hemoglobin in solutions previously reduced by hydro-

STUDIES ON THE OXIDATION AND REDUCTION OF IMMUNOLOGICAL SUBSTANCES.

III. TETANOLYSIN.

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In the preceding papers, certain principles previously established in studies of the oxidation-reduction of blood pigments have been applied to the oxidation-reduction of pneumococcus hemotoxin (1) and the lysin of the Welch bacillus (2). In the present paper, these studies are extended to tetanolysin, the hemotoxin produced by the tetanus bacillus.

The hemotoxin, or lysin, of the tetanus bacillus was first recognized by Ehrlich (3, 4) who clearly differentiated it from the true killing toxin. Much of the lysin-inhibiting or lysin-neutralizing effect of normal sera (3-7) can be referred to the lipoid constituents (8, 9, 10, 13) although several investigators (11, 12) have reported that certain protein constituents are also concerned.

The antibodies of tetanolysin and tetanospasmin have been differentiated by later workers on much the same grounds as those of Ehrlich. Further evidence of the specificity of the tetanus hemotoxin is furnished by the fact that it is not neutralized by the antihemotoxin produced by immunization with the lysins of *Pneumococcus*,¹ *staphylococcus* (14), and of other, anaerobic bacteria (15).

Tetanolysin has been used to illustrate the rate of combination of toxins and antitoxins, in so called "*Heilversuche*" experiments (16, 17); to demonstrate the effect of fractional addition of toxins in toxin-antitoxin mixtures (19); and in a number of other fundamental investigations. The physical-chemical relations involved in the reactions by which the toxin is destroyed, and the effect of temperature upon the rate of hemolysis have also been studied (4, 19, 20, 21, 10). As shown in the preceding paper (2) the activity of tetanolysin is influenced to a much less marked degree by differences in the pH of the hemolysis test system, than is the Welch lysin.

¹ Unpublished experiments of our own.

which is liberated in the medium only upon disintegration of the bacterial cells.

The lysin content of both the plain broth and the glucose broth cultures remained surprisingly constant in cultures varying in age from 16 hours to 12 days. (The pH of the cultures, if measured with a minimum exposure to air, was approximately 6.5.) The presence of glucose influenced the lysin production to a very slight degree; though when any difference existed, it was in favor of the glucose broth culture. In similar experiments with the Welch bacillus a greater lysin content was obtained in the absence of glucose. One would naturally expect the presence of glucose to alter the products of growth of the gas bacillus to a greater extent than it would in the case of the tetanus organism. However, the lower yield of lysin in glucose cultures of the gas bacillus cannot be referred entirely to the influence of the presence of the fer-

TABLE II.
Heat Lability of Tetanolysin.

Amount of lysin.	Original lysin (titre).	Hemolysis by lysin heated for			
		10 min. at 55°C.	10 min. at 60°C.	2½ min. at 65°C.	10 min. at 65°C.
cc.					
0.5	++++	++++	++++	++++	—
0.2	++++	++++	++++	++++	—
0.1	++++	++++	+++	++++	—
0.05	++++	++++	++++	++++	—
0.03	++++	++++	±	+	—
0.02	++++	++++	—	—	—
0.01	++++	++++	—	—	—
0.002	++	±	—	—	—
0.001	±	—	—	—	—

mentable sugar upon the direction of the metabolism of the bacteria. These cultures become quite acid and the precipitation of the lysin or other effects of acidity must also be considered as factors.

Heat Lability of Tetanolysin.

Experiments were made to compare the lability of the tetanus hemotoxin with the lability of the other hemotoxins studied in this series of papers. The lysin was heated in narrow thin walled tubes protected from air. The reaction of the fluids tested, if not exposed to air, was pH 6.6. The tests for active lysin were made immediately after the heating test. The results are given in Table II.

The results of these experiments (Table II) show that tetanolysin is completely inactivated by 10 minutes exposure to 65°C. At this temperature the inactiva-

sulfite. They were also evident in earlier experiments of our own (26) on the determination of hemoglobin in mixtures previously reduced by bacteria in the absence of air. The advantage of low temperature in the preliminary incubation of the hemolysis tests probably depends upon the same principles that made it more desirable to saturate the hemoglobin at low temperature. The lysin can combine with the blood cell at a low temperature just as hemoglobin can combine with oxygen, although the destruction of both the lysin and the hemoglobin by the products of hydrosulfite is retarded to a greater extent by decreased temperature. Possibly also, the lysin after it once combines with the blood cell is less easily oxidized or destroyed, just as ferrous hemoglobin proves more stable after combination with oxygen or carbon monoxide (26).

TABLE I.

Tetanolysin Accumulation in Plain Broth and Glucose Broth at Different Ages of the Culture.

Amount of culture fluid.	Plain broth culture.			Glucose broth culture.		
	Age of culture.			Age of culture.		
	16 hrs.	36 hrs.	12 days.	16 hrs.	36 hrs.	12 days.
cc.						
0.03	++++	++++	++++	++++	++++	++++
0.01	++++	++++	++++	++++	++++	++++
0.004	++	++	+++	+++	++++	++++
0.002	—	—	+	+	+	+
0.001	—	—	—	±	±	±

Tetanolysin Accumulation in Plain Broth and Glucose Broth at Different Ages of the Culture.

The primary object of the following experiment was to determine the period of growth of the culture at which the lysin is produced. It was also desired to determine the stability of the lysin when cultures were held at 38°C. under the usual conditions.

The cultures were grown in infusion broth containing no added glucose and in the same broth containing 1 per cent glucose. The lysin was added to 3 cc. of 2 per cent sheep cells suspended in salt solution; the hemolytic test mixtures were incubated 1 hour at 38°C. and then centrifuged. The results are given in Table I.

The results of these experiments (Table I) show that tetanolysin, like the Welch lysin, is produced early in the growth of the culture. This fact distinguishes both of these lysins from the endocellular hemotoxin of *Pneumococcus*

tion processes proceed rapidly, as about the same amount of lysin is destroyed by $2\frac{1}{2}$ minutes exposure to 65°C. as is destroyed in 10 minutes at 60°C. This degree of heat resistance is considerably higher than that found in tests made under similar conditions with the hemotoxins of the *Pneumococcus*, the Welch bacillus, and El Tor strains of the cholera vibrio. The order of heat lability of the hemotoxins of these different bacteria is as follows: *Pneumococcus*, cholera vibrio, Welch bacillus, and tetanus bacillus.

Inactivation of Tetanolysin by Exposure to Air, and "Reactivation" of the Oxidized Lysin by Treatment with Reducing Agents.

Experiments were made to determine whether the inactive substances formed in aerated tetanus culture fluids included a reversible oxidation product. The methods employed were the same as those

TABLE III.
"Reactivation" of Oxidized Tetanolysin by Reduction.

Tetanolysin.	Hemolytic titre.			
	Amount of lysin.			
	0.10 cc.	0.03 cc.	0.01 cc.	0.005 cc.
Oxidized lysin not treated with $\text{Na}_2\text{S}_2\text{O}_4$.	—	—	—	—
Oxidized lysin after reduction by $\text{Na}_2\text{S}_2\text{O}_4$.	++++	++++	++++	++

used in the preceding investigations. A protocol of a typical experiment is given in Table III to illustrate the results.

The "reactivation" of the hemolytically inactive fluid by treatment with the reducing agent (Table III) can be interpreted as the conversion of a non-hemolytic, reversible oxidation product to the originally "active," reduced lysin. Hence, the inactivation of tetanolysin upon aeration of culture fluids is an oxidation process which yields a reversible product.

The Influence of Temperature upon the Oxidation of Tetanolysin.

The following experiments were made to determine the effect of temperature upon the oxidation of the tetanolysin.

The bacteria-free supernatant fluid of a 12 day glucose broth culture was used in the experiments. 10 cc. portions were exposed to air at 5° , 22° , and 38°C. Samples were taken at the times indicated in the protocol. The pH of the cul-

In any mixture containing hemoglobin and its degradation products, the amount of "active" hemoglobin can be estimated by determining its oxygen- or carbon monoxide-combining capacity. In the same mixtures, the amount of methemoglobin, the reversible oxidation product, may be estimated by reducing the mixture and then determining the increase in oxygen or carbon monoxide capacity. This increase in "activity" can be referred to the reduction of "inactive" methemoglobin to "active" hemoglobin. The detection of other degradation products, such as globin and hematin, requires data on the initial content of hemoglobin in the original fluid before any deterioration has occurred. With these data obtained (the initial "total hemoglobin"), the further degradation products (such as globin and hematin) are estimated by subtracting the sum of

TABLE V.

Mixtures of "Active" Tetanolyisin and Its "Inactive," Reversible Oxidation Product in Culture Fluids Exposed to Air at Different Temperatures.

Amount of culture fluid.	Lysin in untreated fluids (before treatment with reducing agent).				Lysin in fluids after treatment with reducing agents.			
	Unexposed culture fluid.	Culture fluid exposed to air for 24 hrs. at			Unexposed culture fluid.	Culture fluid exposed to air for 24 hrs. at		
		5°C.	22°C.	37°C.		5°C.	22°C.	37°C.
cc.								
0.2	++++	++++	++++	—	++++	++++	++++	++++
0.1	++++	++++	++++	—	++++	++++	++++	++++
0.05	++++	++++	++	—	++++	++++	++++	++++
0.04	++++	++++	—	—	++++	++++	++++	++++
0.03	++++	++++	—	—	++++	++++	++++	+++
0.02	++++	+++	—	—	++++	++++	+++	++
0.01	++++	+	—	—	++++	++++	+++	+
0.005	+++	—	—	—	+++	+++	+	—
0.003	+	—	—	—	++	—	—	—

the hemoglobin and methemoglobin which are present in the deteriorated mixture, from the initial amount of hemoglobin in the undeteriorated fluid. In the case of mixtures of the different modifications of active hemotoxins, the principles involved are exactly the same.

In examination of the lysins, the first data required are measurements of "total lysin." These are obtained by determining the "active lysin" content in undeteriorated culture fluids. The fluid of a young culture, or a fluid which has been protected from deterioration, is titrated for "active lysin" both before and after treatment with a reducing agent. If the lysin content is not increased by the reduction treatment, it is assumed that all of it is present in the "active" form, and that the values obtained represent the "total lysin" produced by the

ture after exposure to air was 7.2. The hemolysis tests were made with 3 cc. of blood cells suspended in salt solution. The results are given in Table IV.

The data in Table IV illustrate the relative rates of oxidation of tetanolysin at different temperatures. As might be expected, the lysin is oxidized much more rapidly at the temperature of the body than at room temperature, being almost entirely destroyed after 11 hours exposure to air at 37°C. The oxidation proceeds much more slowly at the temperature of the ice box.

Attempt to "Reactivate" Heated Tetanolysin by Treatment with Reducing Agents.

In the preceding papers (1, 2) it has been shown that the heat-inactivated products of the hemotoxins of *Pneumococcus* and of the Welch bacillus cannot be "reactivated" by reducing agents. This obtained in tests made with lysins which had been heated the minimum amount required for their complete inactivation. Tests were made to determine if the same relation holds true in the case of tetanolysin.

The results of these experiments were the same as those obtained in analogous experiments with the other lysins. If inactivated by heat, neither the "reduced" active lysin nor the "oxidized" lysin can be "reactivated" subsequently by treatment with sodium hydro-sulfite. Hence, it would seem that the inactivation of tetanolysin by heat is due to the formation of products distinctly different from the oxidation product.

The Distinction between the Reversible and Irreversible Inactivation of Tetanolysin.

The preceding experiments have dealt with two types of inactivation: that due to oxidation, which yields a reversible inactive product, and that brought about by heat, which is irreversible (protein denaturization). It is possible that when culture fluids are aerated, a part of the lysin may also be converted to irreversible products, just as in mixtures of "ferrous" (24), and "ferric" hemoglobin part of the blood pigment may be changed to irreversible products (globin, hematin, etc.). The detection of the irreversibly inactivated lysin corresponds to that of irreversibly inactivated products of hemoglobin.

ference between the original or "total lysin" and the sum of the active lysin plus its reversible oxidation product in the deteriorated fluid may most easily be explained by the formation of other irreversible degradation products which probably arise from the deterioration of the first formed reversible oxidation product. The irreversibly inactivated products of the lysin can be compared to degradation products of hemoglobin, such as globin and hematin, which, unlike methemoglobin, cannot be reduced to the original or "active" blood pigment. While this explanation seems the most likely one, it is necessary to admit the possibility of hydrosulfite failing to reduce completely all of the reversible product.

TABLE VI.

Mixtures of Different Products of Tetanolyisin in Culture Fluids Exposed to Different Conditions.

Previous treatment of culture fluid. 1.	Ly _r (original, active reduced lysin).	Ly _o (inactive reversible oxidation product).	Ly _x (other inactive degradation products).
Not exposed to air.....	200	0	0
Exposed to air for 24 hrs. at 5°C.....	50	150	0
" " " " 24 " " 22°.....	7	143	50
" " " " 24 " " 37°.....	0	33	167

It is evident from the conditions of the above experiment that each of the fluids examined (Table V) originally contained the same amount of the active, or reduced, lysin. The various proportions of the different modifications of tetanolyisin that may be present in the same culture fluids after exposure under the different conditions can be graphically illustrated by collecting the data of Table V in the form given in Table VI.

The "units" of lysin represent the amount of lysin which suffices to give approximately 80 per cent hemolysis of 4 cc. of a 2 per cent suspension of blood cells. These "units" at best are only approximate and are not presented as representing absolute values. They serve, however, to illustrate the principles involved.

The amounts of the different products of the lysin (Table VI) are calculated in exactly the same manner as one would calculate the analogous products of

culture. With the preliminary data at hand, the following measurements are made of the lysin in the deteriorated culture fluids. The "active lysin" is estimated by a titration of the lytic activity of the fluid. The fluid is then treated with the reducing agent and the titration is repeated upon the reduced mixture. As in the case of the blood pigment derivatives, the increase in the "active" substance upon reduction represents the reversible oxidation product. The other degradation products are estimated as the difference between the "total lysin" and the sum of the reduced "active" lysin and the reversible oxidation product.

A number of different mixtures in deteriorated culture fluids have been examined by these methods. The experiment given in Table V represents an examination of the cell-free supernatant of a 12 day culture, portions of which had been exposed to air at 5°, 22°, and 37°C.

The lysin "titrations" of the unexposed culture fluid and of the fluids exposed to air at different temperatures are presented in Table V. The first four columns include the titrations of the fluids prior to treatment with a reducing agent. The results recorded in these columns, therefore, represent the lysin which is in the active or reduced state. The last four columns of the table include the titrations of the same fluids after the reduction treatment. The results of these titrations represent the sum of the active lysin plus the inactive but reversible oxidation product.

A comparison of the lysin activity of the unexposed fluid and of the exposed fluids, before and after treatment with a reducing agent, reveals the following relations. The unexposed fluid exhibits no increase in apparent lysin content upon treatment with a reducing agent, which proves the absence of the reversible oxidation product of the lysin. The value of the lysin titration of the unexposed fluid after the reduction treatment may be taken as "total lysin" present in the culture fluid. In contrast to the unexposed fluid which showed no increase in activity upon reduction treatment, all of the exposed fluids exhibited an unmistakable increase in activity when reduced. The difference between the apparent lysin content of each of the fluids before and after reduction, may be assumed to represent the degree of "reactivation" of the inactive, but reversible oxidation product.

It is evident, however, that only one of the exposed fluids regained the original "total lysin" value after reduction treatment. The failure to regain the original lysin activity, as shown by the dif-

The value of $(Ly_r + Ly_o)$ is obtained by estimation of the active lysin after reduction treatment of the culture fluid, just as the value of $(Hb_{out} + Hb_{ir})$ is obtained after reduction treatment of the hemoglobin solution.

Using the above approximate data of Table VI, it is interesting to present the graphic example shown in Fig. 1 of the variety of mixtures of active lysin and inactive lysin derivatives that may occur in deteriorated tetanus culture fluids.

Fig. 1 illustrates the fact that a series of cultures fluids with the same initial content of lysin may contain at the time of analysis, a variety of mixtures of active lysin and its inactive degradation products. The obvious conclusion, hitherto often overlooked, is that the lysin detected at any one time by the ordinary methods is determined not only by the actual lysin-producing capacity of the culture, but is equally dependent upon the conditions to which the culture fluid has been exposed previous to the time of analysis.

DISCUSSION.

Tetanolysin is inactivated when the culture fluids are exposed to air. The inactive product thus formed is the reversible oxidation product of the "active" reduced lysin since it is converted to the "active" lysin by treatment with the proper reducing agent. In addition to the reversible oxidation product, other inactive degradation products of the lysin are formed upon the aeration of tetanus culture fluids. These substances cannot be "reactivated" by reduction treatment and they probably represent degradation products of the first formed reversible oxidation product.

The methods used in the analysis of mixtures of hemoglobin and its usual degradation products are applicable to the examination of mixtures of "active" lysin and its inactive products. A complete analysis of the lysin products present in a culture fluid requires the following data: (1) the "total lysin" produced by the culture as revealed by the examination of the culture fluid before any of the lysin has deteriorated; (2) the "active" or reduced lysin present in the fluid at the time of examination; and (3) the sum of the "active" reduced lysin plus its inactive reversible oxidation product as revealed by lysin titrations of the fluid after treatment with a proper

hemoglobin from data obtained by the same methods as those used in the above lysin measurements. The analogy is made clear by the following relationships.

Lysins:

$$1(a) \text{ "Total Ly" } = (Ly_r + Ly_o + Ly_z)$$

where Ly_r = reduced or active lysin; Ly_o = the reversible oxidation product of Ly_r ; Ly_z = the irreversibly inactivated products of the lysin.

$$(b) Ly_o = (Ly_r + Ly_o) - Ly_r$$

$$(c) Ly_z = \text{"Total Ly"} - (Ly_r + Ly_o)$$

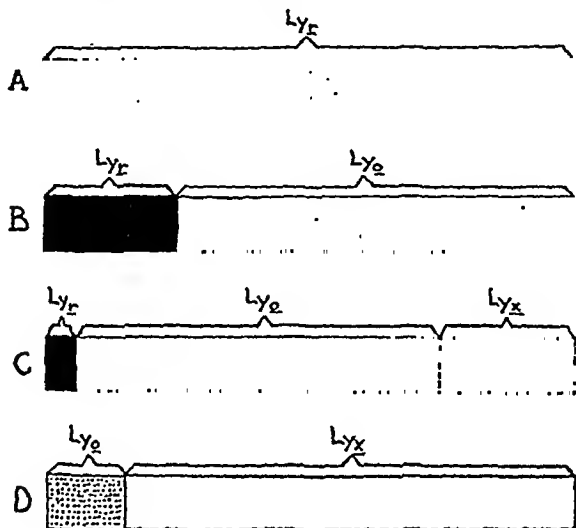


FIG. 1. Examples of the variety of mixtures of active lysin and its inactive modifications that may be present in tetanus culture fluids after exposure to different conditions. A, original culture fluid (not exposed to air). B, culture fluid aerated for 24 hours at 5°C. C, culture fluid aerated for 24 hours at 22°C. D, culture fluid aerated for 24 hours at 37°C.

Hemoglobin:

$$2(a) \text{ "Total Hb" } = (Hb_{ou} + Hb_{ic} + Hb_z)$$

where Hb_{ou} = "active" Hb (Hastings' (24) "ferrous Hb"); Hb_{ic} = "inactive" Hb (MetHb or Hastings' (24) "ferric Hb"), the reversible oxidation product of Hb_{ou} ; Hb_z = the irreversible inactive products of Hb (globin, hematin, etc.).

$$(b) Hb_{ic} = (Hb_{ou} + Hb_{ic}) - Hb_{ou}$$

$$(c) Hb_z = \text{"Total Hb"} - (Hb_{ou} + Hb_{ic})$$

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reducing agent. If these data are at hand, it is possible to estimate the relative amounts of "active" reduced hemotoxin (Ly_r), of its inactive, reversible oxidation product (Ly_o), and of the irreversible degradation products (Ly_z) which cannot be "reactivated" by reduction treatment. It is possible that the irreversible degradation products may be formed by the deterioration of the reversible oxidation product (*i.e.*, $Ly_r \rightarrow Ly_o \rightarrow Ly_z$) in a manner analogous to the formation of globin and hematin by the splitting of methemoglobin (*i.e.*, $Hb \rightarrow MetHb \rightarrow \text{globin and hematin}$) which is the most usual course of blood pigment destruction.

SUMMARY.

A preliminary study was made of the rate of formation of tetanolysin and of the effect of glucose upon the lysin production. The heat lability of tetanolysin was next compared with that of the lysins of other bacteria. Finally the methods used to estimate the relative amounts of "active" hemoglobin and its "inactive" derivatives in deteriorated solutions of blood pigments were applied to the differentiation of the various derivatives of "active" tetanolysin present in deteriorated culture fluids.

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EXPERIMENTAL.

Methods.

Strains of Hemolytic Streptococci.—Two strains of hemolytic streptococci were used in most of the experiments. Strain M was isolated by Dr. William Moss from the tissues of the neck of a child suffering from cellulitis. Strain D is a "scarlet fever strain" which has been used in the preparation of "Dick toxin." Both of these strains produce wide zones of hemolysis in less than 24 hours when grown on meat infusion blood agar plates. Other strains from the collection of the Hospital of The Rockefeller Institute were included in a number of experiments.

Hemolysis Tests.—In the usual test for hemolytic properties of streptococci in liquid systems, the whole culture is added to a suspension of red blood cells. In the present investigation, however, it was necessary to make the actual hemolysis tests with bacteria-free fluids, in order to avoid the possibility of bacterial growth and actual elaboration of lysin during the hemolysis test itself.

In view of the marked instability of the lysin, the manipulation of the culture fluid preceding the measurements of the lysin activity was kept constant throughout all of the experiments. The cultures were grown in broth under vaseline seal. Representative samples were removed and put into narrow tubes, sealed with vaseline, then centrifuged for 45 minutes at high speed. The supernatant fluids used in the tests were added to the blood cell suspensions immediately after the centrifuging process.

The hemolysis tests were made by the same methods as in the preceding studies, and consisted in "titrations" of the minimum amount of the test fluid which suffices for hemolysis of a constant volume of red blood cells. Although there are theoretical objections to this method of measurement of lysin content, the data obtained seem sufficiently accurate for the purposes of the present investigation. In a number of protocols, the results of the lysin "titrations" are recorded in terms of lysin "units." In these instances, the lysin "unit" represents the amount of lysin required to cause 80 per cent complete hemolysis of a constant volume of red blood cells. It is understood that these "units" are not presented as absolute values.

The symbols introduced in the preceding paper (3) are used to distinguish between the different modifications of the lysin; *i.e.*, Ly_r = the "active" lysin present in fluids before treatment with the reducing agent; Ly_o = the oxidized lysin which, although itself "inactive," can be converted to the originally "active" substance by reduction treatment; Ly_x = irreversibly inactivated lysin.

The "Spontaneous Deterioration" of Streptolysin and "Reactivation" of the Inactive Lysin by Treatment with Reducing Agents.

Tests were made of the possibility of "reactivating" deteriorated streptolysin by the reduction treatment employed in the preceding

STUDIES ON THE OXIDATION AND REDUCTION OF IMMUNOLOGICAL SUBSTANCES.

IV. STREPTOLYSIN.

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INTRODUCTION.

The preceding papers (1-3) of this series include reports on the oxidation-reduction of the "hemotoxins" of pneumococci and of Welch and tetanus bacilli. The present paper reports a study of the "spontaneous deterioration" of streptolysin.

Streptolysin may be considered as a substance possessing hemolytic properties, which is produced during the active growth of certain streptococci in a suitable culture medium. This hemolytic action of streptococci has been the subject of many investigations during the past 25 years (4-31). The facts of essential importance in the establishment of the nature of the lysin can be obtained from the studies of Ruediger (24, 25), von Hellens (31), and Meader and Robinson (21).

Although the fact has been disputed in the literature, the actual hemolytic powers of the free lysin are independent of the presence of streptococcus cells. The lytic substance is extremely labile and under the usual conditions rapidly loses its hemolytic property. The change is commonly termed a "spontaneous deterioration," since the loss in activity has been considered to be independent of the conditions imposed during the storage of streptococcus culture fluids.

The literature (5, 8, 19, 22, 16, 24, 25) furnishes little convincing evidence that streptolysin is an antigen. However, the similarity between the "spontaneous deterioration" of the streptococcus lysin and the oxidation of the antigenic lysins of *Pneumococcus* and tetanus justifies the inclusion of the present paper in this series of studies on the oxidation and reduction of immunological substances.

and sealed with vaseline; a second series was placed in Erlenmeyer flasks which exposed a large surface of the fluid to the air.

Both series were then placed in the incubator at 38°C., the usual lysin "titrations" were made at the end of 6, 12, and 24 hours.

The results of one series of tests with Strain M are given in Table II.

In Table II, the results presented in the first three columns show that while the lysin gradually "deteriorates" to inactive products when protected from air by a heavy vaseline seal, free exposure to air causes a much more rapid inactivation.

The last three columns of Table II, which record the apparent lysin content of the fluid after treatment with the reducing agent, represent

TABLE II.

Influence of Exposure to Air upon the "Spontaneous Deterioration" of Streptolysin.

Ly _r (Lysin activity before treatment with hydrosulfite).				Ly _r + Ly _o (Lysin activity after treatment with hydrosulfite).		
Amount of culture fluids.	Original culture fluid (12 hr. culture).	Fluid exposed to air 6 hrs. at 38°C.	Fluid stored in sealed tube 6 hrs. at 38°C.	Original culture fluid.	Fluid exposed to air 6 hrs. at 38°C.	Fluid stored in sealed tube 6 hrs. at 38°C.
cc.						
1.0	++++	++	++++	++++	++++	++++
0.8	++++	±	++++	++++	++++	++++
0.6	++++	—	++++	++++	++++	++++
0.4	++++	—	++++	++++	++++	++++
0.2	+++	—	++	++++	++++	++++
0.1	+	—	—	++	++	++
0.05	—	—	—	—	—	—

the sum of the active lysin (Ly_r) plus its inactive, but reversible oxidation product (Ly_o). It is important to note that the inactive products formed from streptolysin either in sealed tubes or under conditions of free exposure to air can, in each instance, be converted to the original, active lysin by reduction treatment. Since the inactive products formed are apparently the same, it is fair to conclude that the inactivation processes consist of the same type of reaction—*i.e.*, oxidation processes are responsible for the "spontaneous deterioration" of streptolysin in sealed tubes as well as in aerated fluids. Whether the oxidizing agents involved in the actual oxidations of the lysin are

investigations. The results of a typical experiment are presented in Table I.

As shown in Table I, "spontaneously deteriorated" streptolysin is "reactivated" by the reduction treatment successfully employed in the "reactivation" of the hemolytically inactive oxidation products of the previously studied bacterial lysins. It seems, therefore, that these inactive products (as in the case of the pneumococcus, Welch, and tetanus lysins) represent reversible oxidation products which can be converted by reduction, to the original, hemolytically active lysin. Since inactivation can be brought about by aeration and the

TABLE I.

"Reactivation" of "Spontaneously Deteriorated" Streptolysin by Treatment with Reducing Agents.

Amount of fluid.	Hemolytic activity of "spontaneously deteriorated" fluids of streptococcus cultures.	
	Not treated with hydrosulfite.	Treated with hydrosulfite.
cc.		
1.0	—	++++
0.6	—	++++
0.4	—	++++
0.2	—	++++
0.1	—	++++
0.05	—	++

inactive products in turn can be "reactivated" by reduction, it appears that the long discussed "spontaneous deterioration" of streptolysin is simply a reversible oxidation-reduction process.

The Influence of Exposure to Air upon the "Deterioration" of Streptolysin.

In the experiments described below, a comparison is made of the relative rates of "deterioration" of streptolysin when the culture fluid is stored in sealed tubes and in shallow layers freely exposed to air.

Cultures of two strains of hemolytic streptococci were grown in infusion broth in tubes sealed with vaseline. After 12 hours incubation, uniform portions of the culture were removed; one series of each strain was placed in narrow tubes

Sample (6) consisted of a sealed tube of the sterile culture fluid. A second series exactly the same as that described above was prepared with the exception that methylene blue was added to each of the tubes.

Series I and Series II were now placed at 38°C. At the end of 6, 12, 24, 48, and 72 hours, lysin titrations were made on Series I, and observations of the degree of reduction of methylene blue were made on Series II.

In all experiments of the type described above the lysin proved most stable in the fluids in which the reduction of the dye was most complete and most persistent. Thus, as indeed might be expected, conditions least favorable for the conversion of the colorless dye to its colored oxidation product likewise proved to be least favorable for the conversion of streptolysin to its hemolytically inactive oxidation product.

In the unsealed tubes, the reduction of methylene blue was transient; upon the cessation of growth activity of the bacteria, the dye was more or less rapidly converted to its colored oxidation product. In these tubes, in which both dye and lysin were finally oxidized, the time at which the maximum amount of lysin was in the active or reduced state, coincided with the time at which the maximum amount of the dye was in the colorless or reduced condition. Methylene blue, however, is much more readily maintained in the reduced condition than is the active streptolysin.

In the fluids which were sealed from air, the greater tendency of the lysin toward oxidation limited the application of the comparison of the persistence of methylene blue reduction with the persistence of active lysin. Even the sterile culture fluid containing no bacteria possessed sufficient reducing power to maintain the dye in the reduced state, if protected from air by a vaseline seal. Thus, it is evident that streptolysin is a substance much more readily oxidized than is methylene white, since the conversion of the colorless dye to its oxidation product was prevented in systems in which the active (or reduced) lysin was more or less rapidly converted to the inactive oxidation product.

Heat Lability of Streptolysin.

That streptolysin is a heat-labile substance has been well established in the literature (15, 14, 31, 27, 18, 20, 5, 31), but the relative degree of heat lability varied in the results of different investigators. Experiments on the heat lability

the same in each case, is, of course, not proved. The essential difference, however, in the "deterioration" of streptolysin under the two sets of conditions, seems to be a difference in the rate of the reaction.

A third point of interest revealed in Table II is that in spite of the greater "deterioration" of the lysin in the aerated series, treatment with the reducing agent restores the same original lysin activity to both the sealed and aerated fluids. Thus, while in the aerated fluids much more of the active lysin has been converted to its inactive, reversible oxidation product than in the fluids held in the sealed tubes, the sum of the active lysin plus its reversible oxidation product remains the same in each instance. Apparently, under the conditions of this experiment, none of the lysin was destroyed to non-reversible, inactive products.

The results of the tests with Strain D are not recorded in Table II. The difference in stability of streptolysin in cultures of the two strains (D and M) is worthy of note, as it was constantly evident throughout the entire investigation. For example, in the above experiment cultures of Strain M lost more of the original lysin activity after 6 hours storage at 38°C., than did Strain D after 20 hours. However, the same relation between the rates of inactivation of lysin in the fluids protected from and exposed to air were evident with the latter strain as that recorded above for the more labile cultures of Strain M.

Comparison of Lysin Stability and Methylene Blue Reduction in Culture Fluids Exposed to Different Conditions.

The object of the following experiment was to compare the stability of the active (or reduced) streptolysin in different systems with the methylene blue-reducing power of the same systems.

Samples of a 12 hour broth culture of Strain M were placed under the following conditions: (1) freely exposed to the air in a shallow layer in an Erlenmeyer flask; (2) a small surface exposed to the air (the height of the cylinder of fluid was 150 mm. while the surface exposed to air was only 15 mm. in diameter); (3) sealed from air by a heavy layer of vaseline; here, too, the surface of the broth was only 15 mm. in diameter while the depth was 150 mm. Samples (4) and (5) were, respectively, the same as (2) and (3), with the exception that a suspension of *B. coli* was added to each, to furnish additional reducing action.

were made at the end of 6, 12, 24, and 48 hours. A constant volume of 4.0 cc. was maintained in the hemolysis systems; and the following increments of diluted culture fluid were tested: 0.80, 0.60, 0.50, 0.40, 0.35, 0.30, 0.250, 0.225, 0.175, 0.150, 0.125, and 0.100 cc. The hemolysis tests were incubated 45 minutes and then centrifuged.

The results of this experiment are presented in Fig. 1. Each "unit" of lysin represents the amount of lysin which causes approximately 80 per cent hemolysis of 4 cc. of a 2 per cent suspension of sheep cells. These "units" are not presented

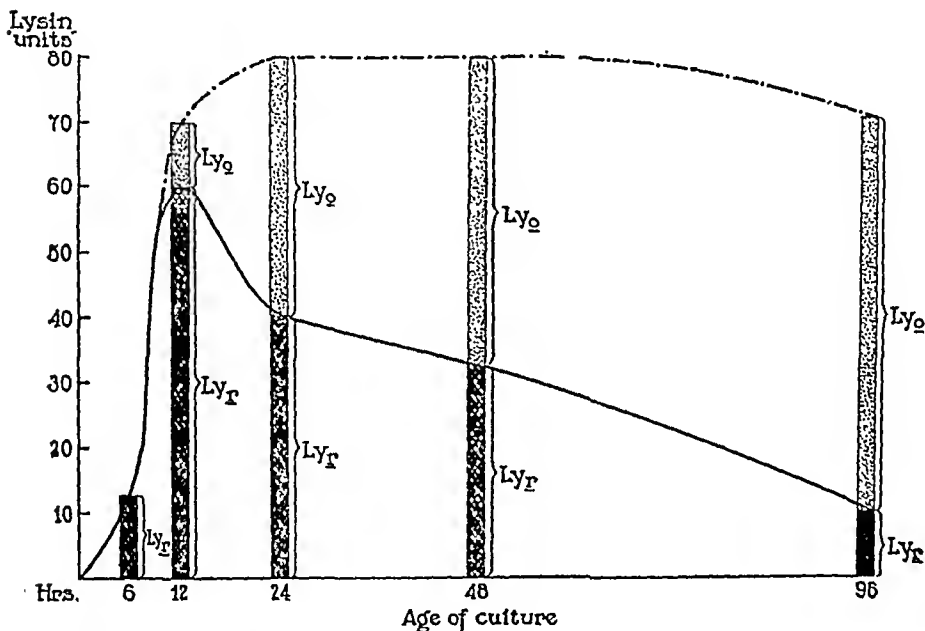


FIG. 1. Relative amounts of active and deteriorated lysin in streptococcus cultures at different ages of the culture.

The black unbroken line represents the active, reduced lysin (Ly_r); the broken line represents the sum of the active lysin plus its hemolytically inactive, but reversible, oxidation product ($Ly_r + Ly_o$). The black shaded areas represent the amount of active lysin (Ly_r) in the fluid at the time of the tests; the stippled areas represent the amount of the reversible oxidation product (Ly_o).

as absolute values, but are used merely as a convenient index for the approximate comparison of the relative lysin content of the fluid at different stages of growth of the culture.

In Fig. 1, the continuous line and the shaded areas represent the amount of lysin in the active state at the time of the test, the results having the character of those which have been reported for years in

of the streptolysin in the fluids used in the present studies were made to furnish data required in the next experiment. The fluids were heated in sealed tubes in the absence of air; the reaction of the fluids was approximately pH 6.8. The results of these experiments showed that the lysin, when heated under these conditions, was completely inactivated by 5 minutes exposure to 55°C., while over half of the lysin was destroyed by 10 minutes exposure to 50°C. Thus, as shown by comparison with the results in the preceding studies (1-3) streptolysin possesses about the same degree of heat lability as the lysins of the Welch bacillus and the El Tor cholera vibrio; it is somewhat less heat-labile than pneumococcus hemotoxin, and somewhat more thermolabile than the lysin of the tetanus bacillus.

Attempt to "Reactivate" Heat-Inactivated Streptolysin by Treatment with Reducing Agents.

The heated fluids used in the experiments were exposed to 55°C. for 5 minutes (the minimum heating treatment found in the preceding experiment to effect the complete inactivation of the lysin). The "oxidized" or "deteriorated" lysin consisted of the centrifuged supernatant of a 5 day culture of the same strain. In both cases, the cultures had been grown and stored in vaseline-sealed tubes. The heating tests were likewise made in vaseline-sealed tubes.

The results of a number of experiments proved that heat-inactivated streptolysin cannot be "reactivated" by treatment with the reducing agent, sodium hydrosulfite. Thus, the heat inactivation of streptolysin is due to the formation of inactive products distinctly different from the reversible oxidation products formed during the "spontaneous deterioration" of the lysin.

Relative Amounts of Active and "Deteriorated" Lysin in Different Stages of Growth of Streptococcus Cultures.

Previous studies of streptolysin have furnished measurements not of actual lysin production, but of its relative stability at the time of the tests. In the experiment described below, streptolysin production and "deterioration" during different periods of growth of the culture were followed in a number of experiments by means of methods whereby the inactive reversible oxidation product of the lysin is detected.

Tubes containing 100 cc. of infusion broth sealed with vaseline were inoculated with 0.4 cc. of a 6 hour culture of Strain M. "Titrations" of the lysin

In Fig. 1, it is also significant that while the amount of active lysin rapidly diminishes, the sum of the active lysin and its inactive, reversible "deterioration" product ($Ly_r + Ly_o$) remains constant for several days. In most of our experiments, the sum of these two products remained the same for 5 or 6 days. The fact that still older culture fluids were found not to regain all of their original active lysin content after the reduction treatment can be explained by the formation of irreversible degradation products (Ly_z) which cannot be converted by reduction to the originally active lysin.

The Influence of Yeast Extract in the Culture Medium upon the Production and Stability of Streptolysin.

As suggested by Meader and Robinson's (21) work, one of the constituent which is required for streptolysin production bears certain resemblances to the so called "accessory substances." The work of Avery and his associates (32), as well as that of other investigators, has demonstrated the marked effect which yeast extract may have upon microbial activity. Experiments on the influence of yeast extract upon the formation and stability of streptolysin seemed especially pertinent to the present investigation, since certain constituents of yeast extract had been found in previous studies (33) to have an intimate relation to the oxidation-reduction activities of different bacteria.

In experiments of the type described below, comparisons were made of the lysin produced in cultures grown in "unfiltered" muscle infusion broth with that produced in the same broth enriched by the addition of yeast extract. In the comparison of lysin production in different culture media, the methods developed in the preceding papers offer certain definite and obvious advantages since they furnish values more closely approaching the "total lysin" actually produced in the tested medium.

"Unfiltered" Muscle Infusion Broth.—Finely minced, fat-free, heart muscle was infused in slightly acidified tap water for several hours and then gradually

the whole culture is added to a suspension of blood cells, in place of the bacteria-free fluids used in the hemolysis tests in this study. Under these conditions, with a reasonably young, whole culture the danger of failure to detect lytic capacity is much more remote than would appear from the results of the preceding experiments, in which all tests were made with the bacteria-free culture fluids.

the literature. The broken lines and stippled areas, however, represent the hemolytically inactive oxidation product of the original lysin and contribute data impossible to obtain by measurements of active lysin alone. Hence, the broken line in Fig. 1 presents a picture which more closely approaches the actual progress of streptolysin production than do any of the previous "curves of lysin production."

While there is little or no "deteriorated" lysin present during the period of maximum growth of the streptococci, the "deterioration" has begun, in this particular culture, by the time of the 12 hour test. Moreover, it is evident in Fig. 1 that the amount of active lysin may begin to diminish before the maximum amount of "total lysin" ($Ly_r + Ly_o$) has been liberated into the medium. This is an important fact and demonstrates that, at least with certain strains of streptococci, the measurement of lysin by the usual method never furnishes the value of the total amount of lysin actually elaborated by the culture. This has been especially true with our Strain M, with which it has never been possible to obtain samples of culture fluid in which there was no "deteriorated" lysin—*i.e.*, fluids in which Ly_r was not less than $(Ly_r + Ly_o)$. We have conducted a number of experiments of the type described above with different strains of streptococci. The fact that with some strains the lysin "deterioration" begins before the liberation of the maximum amount of "total lysin" while with other strains the lysin proves more stable, is evidence that the usual measurements of active lysin cannot furnish a proper basis of comparison of actual lysin production by different streptococci.¹

¹ If practically all of the lysin had "deteriorated" in the fluid tested, the usual method of lysin measurement might lead one to believe that the culture tested was totally devoid of lysin-producing powers, or at least that no lysin had been produced in the tested culture medium.

It would be a mistake however, to emphasize this possibility as a likely source of error in determining whether or not a particular strain of streptococcus is a "hemolytic streptococcus." As a matter of practice, the criterion used in the separation of "hemolytic" streptococci from "non-hemolytic" strains is the production of distinct wide zones of hemolysis during the early growth of the colonies on a blood agar plate (the "beta hemolysis" of Smith and Brown). Where hemolysis in liquid systems is used as a descriptive or systematic criterion

The lysin measurements were made by the methods used in the preceding experiments. Human blood cells were used in these experiments.

The results of an experiment performed as described above are presented in graphic form in Fig. 2. The same lysin "units" are employed as those used in the presentation of the experiment illustrated in Fig. 1.

The results of these experiments (Fig. 2) show that the addition of yeast extract to the medium enhances lysin formation, since it causes an increase in both the rate of lysin elaboration and the "total lysin" production. However, it is more pertinent to the present study to observe that the lysin after its elaboration "deteriorates" much more slowly in the enriched medium. For example, although much of the lysin formed in the unenriched culture medium "deteriorated" between the 12 and 24 hour tests, during the same period in the yeast extract broth, none of the lysin was converted to inactive oxidation products.

While certain constituents of yeast extract may have a specific effect upon the formation of streptolysin, the differences observed between the media used in the above experiment could also be explained by known effects of yeast extract upon bacterial growth activity. The increased rate of lysin formation as well as the greater total production of lysin may be due to a more rapid rate of growth of the streptococci in the enriched medium. It is probable that the deterioration of the lysin in any medium depends upon the loss in the reducing activity which was maintained during the period of active bacterial growth. If the yeast extract enables the streptococci to retain their life or growth activity for a longer period, the increased stability of the lysin is easily explained by the more persistent reducing conditions maintained in the yeast broth.

The difference in stability of the lysin in the two culture media offers another example of the advantages of the method of lysin measurement described. In the case of the unenriched broth, with certain strains of streptococci, a significant amount of active lysin is always converted to inactive products before the total amount of lysin actually elaborated has been liberated into the medium. Since in the yeast extract cultures the lysin proved more stable, it is obvious that a fair comparison of the actual production of lysin in the two media must take into account the inactive products formed by the deterioration of originally active lysin.

brought to a boil. Peptone and salt were added to the decanted infusion, and the broth, still slightly acid, was autoclaved for 10 minutes. The broth was adjusted to pH 7.8, let stand for several hours, and the clear supernatant siphoned off. This medium was finally sterilized in the autoclave for 8 minutes in tubes containing 100 cc. of the medium. After sterilization, a layer of sterile vaseline was added to the hot broth.

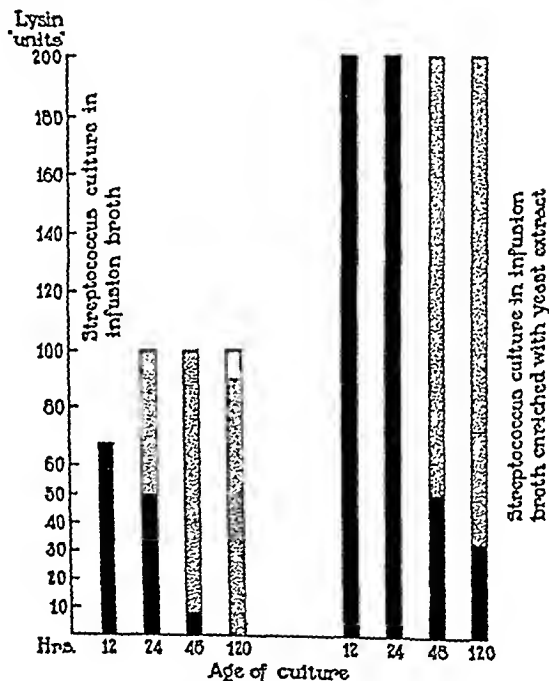


FIG. 2. The influence of yeast extract in the culture medium upon the production and stability of streptolysin.

The black shaded areas represent active (or reduced) lysin (Ly_a). The stippled areas represent the hemolytically inactive, reversible oxidation product (Ly_b) of the original or reduced lysin. The white area represents hemolytically inactive degradation products (Ly_c) of the lysin which cannot be "reactivated" by reduction treatment.

Broth Enriched with Yeast Extract.—Yeast extract broth was prepared by adding 7.5 cc. of yeast extract to each of several tubes of the above described meat infusion broth. The yeast extract was furnished by Dr. O. T. Avery of the Hospital of The Rockefeller Institute. The method of preparation of the yeast extract has been described in previous papers (34).

siderably; during the first 5 days it was approximately 25°C.; between the 5th and 21st days, it reached 32°C. on several occasions. The 140 day sample unfortunately was exposed to several prolonged periods of hot weather in which the temperature was frequently 35°C.

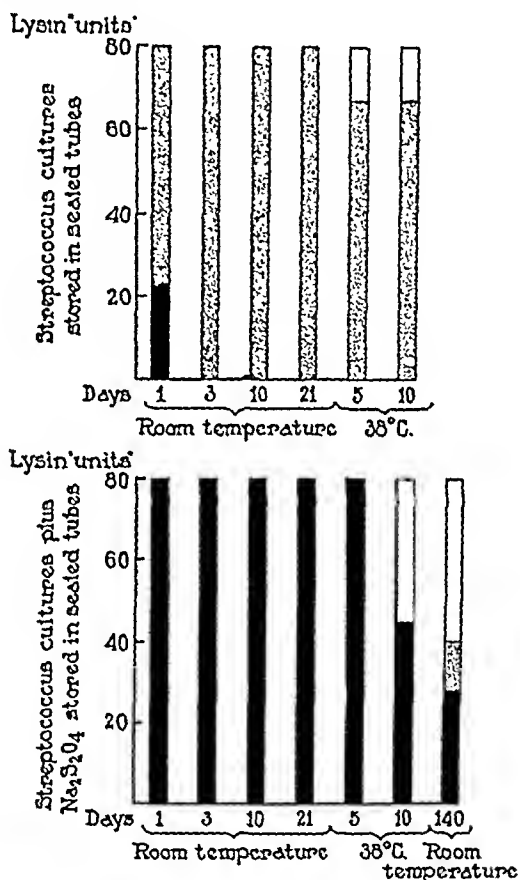


FIG. 3. Inhibition of the "spontaneous deterioration" of streptolysin by storing the culture fluids in a sealed system containing sodium hydrosulfite.

The black areas represent the reduced or active lysin (Ly_r); the dotted or stippled areas represent the hemolytically inactive reversible oxidation product of the lysin (Ly_o); the white areas represent further, inactive degradation products (Ly_z) which cannot be "reactivated" by reduction treatment.

Lysin Measurements.—The tubes of culture fluid were centrifuged and the bacteria-free supernatants used in the tests. 2 cc. of the test fluid were placed in each of two tubes; one of the tubes already contained 2.0 cc. of 0.1 M phosphate solution; 2.0 cc. of 1.0 per cent $Na_2S_2O_4$ in 0.1 M phosphate solution were

The Inhibition or Prevention of the Usual "Spontaneous Deterioration" of Streptolysin by Storing the Culture Fluids in a Sealed System Containing Sodium Hydrosulfite.

From analogous studies on hemoglobin (35), it seemed possible that the "spontaneous deterioration" of the bacterial lysin might be prevented if reducing conditions were imposed during its storage. To test the validity of this assumption, sodium hydrosulfite was added to streptococcus culture fluids in sealed tubes; and the stability of the lysin under these conditions was compared with the stability of the lysin in the same culture fluids to which no reducing agents had been added.

Cultures of Strain M grown in unenriched muscle infusion broth were chosen, as the lysin of this strain had proved most labile throughout all of the preceding experiments.

Portions of the streptococcus culture fluid, taken after stirring the culture, were assumed to be representative samples containing equal amounts of the lysin. The following series of tubes were then prepared:

1. *Culture Fluid Alone*.—4 to 5 cc. of the culture fluid were placed in narrow agglutination tubes so that the tubes were filled to within $\frac{1}{2}$ to $\frac{3}{4}$ of an inch from the top.

2. *Culture Fluid Plus Hydrosulfite*.—Tubes containing 25 mg. of $\text{Na}_2\text{S}_2\text{O}_4$ had previously been sterilized in hot air at 140°C . To these tubes, measured 5 cc. portions of the culture were added, which gave a concentration of 0.5 per cent of hydrosulfite.

pH of the Fluids.—The reaction of the culture fluid alone was approximately pH 6.6. The addition of the hydrosulfite resulted in a drop in pH, so that the culture plus hydrosulfite was about pH 5.9.

In an attempt to obtain a series containing the reducing agent but at the pH of the culture fluid alone, sterile NaOH was added to six of the hydrosulfite series. Unfortunately, through an error, too much alkali was added, and the final reaction at which these tubes of fluid were stored was about pH 9.0.

Sealing of the Tubes.—The tubes were sealed with care to make the exclusion of air as absolute as possible. The narrow tubes were completely filled with melted sterile vaseline immediately after the addition of the culture fluid. The excess vaseline escaped through the groove when a sterile, grooved cork stopper was carefully forced into the tube. The cork was finally completely sealed with sealing wax.

Storage of the Fluids.—A series of tubes containing culture fluid alone and tubes containing culture fluid plus $\text{Na}_2\text{S}_2\text{O}_4$ were stored at 38°C .; a second series were stored at room temperature. The temperature of the room varied con-

considerable portion has become inactive. It is possible that the failure of these fluids to retain their original lysin activity is due to some effect of prolonged exposure to high temperatures upon the hydrosulfite, as well as upon the lysin. (During the later part of the storage of the 140 day "room temperature" tube, it was subjected for several days to a temperature of 35°C.) It is significant that the lysin in these instances was converted to irreversible products rather than to the usual reversible oxidation product.

DISCUSSION.

Streptolysin is an extremely labile bacterial product. Under ordinary conditions, its loss of activity is so rapid and so pronounced that the hemolytic property of streptococcus culture fluids is recognized throughout the literature as "*une propriété éphémère*" (13).

In the present paper, it is shown that the hemolytically inactive product formed by this "spontaneous deterioration" can be "reactivated" by treatment with a chemical reducing agent. If the "reactivation" represents the reduction of an inactive, reversible oxidation product to the original active substance (reduced streptolysin), the process involved in the usual "spontaneous deterioration" of streptolysin can be regarded as an oxidation reaction which yields a reversible product.

Free exposure to air is not required for the oxidation of streptolysin. Although aeration of the culture fluid in shallow layers results in a much more rapid inactivation of the lysin, culture fluids held in tubes sealed from air gradually lose their hemolytic activity. The "deterioration" of streptolysin in tubes protected from air is in marked contrast to the stability of the lysins of Welch and tetanus bacilli, which, at least in culture fluids more acid than pH 7.0, remain active for months if the culture tubes are sealed with vaseline. The inactivation of the lysin in sealed tubes is due to the formation of a reversible oxidation product and hence, must be accepted as an oxidation process which yields a product identical or similar to that formed in aerated culture fluids. It is quite possible that the exclusion of air was not absolute in any of our experiments, and in the absence of exact controls of the gas content of the culture fluids, one cannot meet the question of whether or not traces at least of molecular oxygen are required for the oxidation of streptolysin.

added to the other tube. After allowing time for reduction of the second tube, aliquots of these mixtures were used in the lysin titrations. Tests of the first of these mixtures represent measurements of the active lysin alone; the tests of the second of the above mixtures include not only the active lysin but the inactive, reversible oxidation product.

Controls in which uninoculated broth was stored in sealed tubes containing $\text{Na}_2\text{S}_2\text{O}_4$ were included to eliminate the possible formation of hemolytic substances by action of hydrosulfite upon the culture medium itself.

The results of this experiment are presented graphically in Fig. 3. In this figure, the measurements of lysin content of the fluids are recorded in terms of "lysin units," one "unit" representing the amount of lysin required to cause approximately 80 per cent hemolysis of 3 cc. of a 2 per cent suspension of blood cells.

In streptococcus cultures stored in sealed tubes under the usual conditions (*i.e.*, containing no added reducing agent), the active lysin is always soon converted to inactive products. In the experiment presented (Fig. 3) the fluid had become entirely devoid of lytic action after 3 days storage at room temperature. In fact, the "spontaneous deterioration," as might be expected from previous results (Fig. 1) with this especially labile strain, had already begun at the beginning of the storage experiment.

In contrast to the usual rapid "deterioration" in streptococcus cultures, the series in which hydrosulfite had been present during storage retained their entire original lysin activity for at least 21 days storage at room temperature, and for over 5 days at 38°C. Upon addition of the hydrosulfite at the beginning of the storage period, the hemolytically inactive oxidation product already formed in the fluid was reduced back to the original, active lysin. Then after the system was sealed the excess reducing agent maintained conditions which made impossible the usual "spontaneous" deterioration of lysin during the subsequent storage. It is evident that under these conditions (*i.e.*, stored in a sealed system in the presence of an active reducing agent), streptolysin, a classically labile bacterial product, would appear to be a relatively stable substance.

The results obtained with the hydrosulfite fluids after storage for 10 days at 38°C. and 140 days at "room temperature" introduce certain complications which, however, do not detract from the important relation evident in the results already discussed. Although some active lysin still persisted in these fluids, a

time at a value approximating unity. It is probable that the reducing conditions established by the streptococcus cells during the period of maximum growth diminish in intensity upon the cessation of the bacterial growth activity and this failure of the bacterial culture to maintain the strong reducing conditions which obtained during the period of active lysin elaboration is the cause of the conversion of active lysin to its inactive oxidation product (*i.e.*, a decrease in the ratio $\left(\frac{\text{Ly}_r}{\text{Ly}_r + \text{Ly}_o}\right)$). From this point of view, one would expect the conversion of the active lysin to its oxidation product to be inhibited in any system in which reducing conditions are maintained for a longer period. If the ratio of active lysin to total lysin $\left(\frac{\text{Ly}_r}{\text{Ly}_r + \text{Ly}_o}\right)$ be accepted as an index of reducing conditions in the culture fluid, it is interesting to observe that observations of an unusual persistence of active streptolysin in certain cultures (as for example, the yeast extract cultures of our Strain D) indicate that reducing conditions of sufficient intensity are maintained for a longer period in these cultures.

It has been mentioned before that the lysin of streptococcus "deteriorates" in vaseline-sealed culture fluids, while the lysin in culture fluids of pneumococci (36), tetanus (3), and Welch bacilli (2) are relatively stable if similarly protected from air. An explanation of the greater tendency of streptolysin toward "deterioration" involves two factors: either reducing conditions of sufficient intensity are maintained for a longer period in the cultures of the other bacteria, or streptolysin itself is a substance intrinsically more easily oxidized than the other lysins. The importance of the first factor has already been illustrated by the relative stability of the usually labile streptolysin when stored in a sealed system containing an added chemical reducing agent. A true evaluation of the second factor must depend upon measurements of the reducing intensities obtaining in the respective culture fluids. However, the fact that streptolysin is converted to its oxidation product in systems of sufficient reducing power to prevent a similar conversion of methylene white indicates that the lysin itself is a relatively easily oxidized substance.

The usual method for measuring bacterial lysins in culture fluids detect only the lysin which is in the active state at the time of the test. The methods previously employed in the studies (2, 3) of the Welch and tetanus lysins furnish in addition to measurements of active lysin, measurements of total lysin production and of the ratio of active to total lysin.

Applications of these methods have furnished a picture more complete than those existing heretofore of the actual course of lysin production and of lysin "deterioration" at various stages of growth of different strains of streptococci. The most important facts revealed were the following: (1) the content of active lysin (Ly_a) frequently decreases before the maximum amount of total lysin ($Ly_r + Ly_o$) is liberated into the medium; (2) the ratio $\left(\frac{Ly_r}{Ly_r + Ly_o}\right)$ varies with different strains and with different conditions to which the culture is subjected. These two facts in themselves are sufficient evidence that methods detecting only active lysin (Ly_a) cannot furnish a proper basis for comparisons of actual lysin production, either in experiments with different strains under the same conditions or in experiments with the same strain under different conditions.

The "spontaneous deterioration" of streptolysin, like the usual "spontaneous deterioration" of hemoglobin, consists in the conversion of an active substance to its inactive reversible oxidation product. As previously shown for hemoglobin (35), the formation of the inactive oxidation product is inhibited or wholly prevented in sealed systems containing an active reducing agent. For example, with one of the more labile strains studied, all of the active lysin "deteriorates" to inactive products in 2 or 3 days when stored at room temperature in sealed tubes containing no added reducing agent; on the other hand, in sealed tubes to which hydrosulfite had been added, the same culture fluids were stored for more than 21 days without loss in the apparent content of active lysin and without formation of a detectable trace of the inactive "deterioration" product. Apparently, therefore, if reducing conditions of sufficient intensity be maintained in the culture fluid, the ratio of active lysin to total lysin (*i.e.*, $\frac{Ly_r}{Ly_r + Ly_o}$) remains constant for a considerable

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SUMMARY.

The "spontaneous deterioration" of streptolysin has been studied by the methods already used in investigations of the oxidation-reduction of blood pigments and of the lysins of other bacteria. From the results of this study, it may be concluded that the commonly observed "spontaneous deterioration" of streptolysin consists in the conversion of the originally active lysin to a hemolytically inactive oxidation product. This process is a reversible one, and the activity of the lysin is restored by reduction.

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properties became possible as soon as more elaborate procedures⁶ were developed; namely, the isolation of cells in pure cultures, the use of both nutrient and preservative media, the maintenance of a constant composition of the latter, the measurement of the rate of growth, the cinematographic recording of cell locomotion and of the movements of the protoplasmic constituents, etc. In the experiments which are the subject of the present article, an attempt has been made to ascertain the fundamental properties of the fibroblast and to correlate both its functional state and its changing appearance.

Characteristics of the Colonies of Fibroblasts.

Fibroblasts never grow as isolated units, but form a dense tissue. When a colony is embedded in plasma, the fibroblasts migrate into the new medium without losing contact with the tissue from which they come. The area covered by the colony gradually increases and the cells never scatter through the medium, as macrophages do. On the advancing edge of the tissue, a few isolated cells may be observed, but they are generally united by their processes to neighboring cells. A colony is circular, and the long axis of the cells of its advancing edge is generally parallel to its radius. The fibroblasts remain in intimate reciprocal contact on all sides and multiply actively when packed together. This characteristic probably belongs to all fixed connective tissue cells, as Rous has found that isolated mesenchyme cells in suspension in a medium unite again to form a reticulum.⁷ The aptitude to form a tissue establishes a fundamental difference between fibroblasts and macrophages since the macrophages live as independent units and die if they congregate in dense masses. When two colonies of fibroblasts grow in a single flask at a short distance from one another, they display a tendency to unite their edges. It seems as if the cells of one colony attract the cells of the other, and the rate of growth of the parts of the tissue that are at close range slightly increases until the union of both colonies takes place.

Fibroblasts in pure culture never invade the entire medium. When a small colony is implanted in the center of a flask 50 mm. in diameter,

⁶ Carrel, A., *Physiol. Rev.*, 1924, iv, 1; *Brit. Med. J.*, 1924, ii, 140.

⁷ Rous, P., and Jones, F. S., *J. Exp. Med.*, 1916, xxiii, 549.

THE FUNDAMENTAL PROPERTIES OF THE FIBROBLAST AND THE MACROPHAGE.

I. THE FIBROBLAST.

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PLATES 6 AND 7.

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Tissue cells, like bacteria, must be defined not only by their morphology but also by their physiological properties. A complete knowledge of the biological characteristics of the various cell types would render possible the prediction of the behavior of the cells themselves, as well as their identification. We are still ignorant of these essential properties of the fibroblast and the macrophage, although their anatomy and their staining reactions have been extensively studied during the last 20 years. In his fundamental work on connective tissue, Renaut discovered the characteristic manner in which both types of cells respond to neutral red.¹ His findings were confirmed by Evans and Scott, who ascertained the differential reaction of the fibroblast and the macrophage, not only to neutral red, but also to a number of acid dyes.² The protoplasmic structures of these cells have also been minutely described by Maximow,³ and by Lewis and Lewis^{4,5} in their painstaking studies of mitochondria and neutral red vacuoles in tissues surviving *in vitro*. As these experimenters did not extend their investigations beyond the morphological realm on account of the inadequacy of their techniques, the basal characteristics of the fibroblast have remained unknown. However, a study of these

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² Evans, H. M., and Scott, K., *Carnegie Institution of Washington, Pub. No. 273, Contributions to Embryology*, 1921, x, 3.

³ Maximow, A., *Beitr. path. Anat. u. allg. Path.*, 1902, suppl. 5, 1.

⁴ Lewis, M. R., and Lewis, W. H., *Am. J. Anat.*, 1914-15, xvii, 339.

⁵ Lewis, W. H., *Bull. Johns Hopkins Hosp.*, 1919, xxx, 81.

The section of the culture indicated that nowhere had the fibroblasts mixed with the epithelial cells. The epithelium had grown in a gland-like formation, without being penetrated by the fibroblasts. Generally macrophages do not remain among fibroblasts. They migrate more quickly and scatter freely through the medium far from the colony of fixed cells.

Rate of Growth of the Colonies.

The rate of growth of the colonies under various conditions has been ascertained by a large number of measurements in the course of the last 14 years. In a medium composed of 1 volume of adult chicken plasma and 1 volume of juice from 8 or 10 day old chick embryos and kept at a temperature of 39°C., the colonies double in mass every 48 hours. This means that a weight of protoplasm equal to that of the original colony is synthesized from the medium constituents during this short period of time. The epithelial cells respond also to the presence of embryo juice by a great increase in their activity. But their rate of proliferation never equals that of fibroblasts. In the same cultural conditions, the macrophages cease to multiply. The fibroblast is the fastest growing cell that has so far been isolated. The velocity of cell multiplication depends at first on the age of the animal or the condition of the culture from which the colony is obtained.¹² Fibroblasts removed from an embryo or a young culture multiply *in vitro* more rapidly than those from an older embryo or older culture. But the activity of fibroblasts of different original growth energy and cultivated in identical media has a tendency to become uniform. After they have been cultivated for a few days or weeks in the same substances, they proliferate at the same speed.¹³ The rate of growth of a colony at a given instant is a function of its activity at the preceding instant, and of the concentration of the growth-activating and inhibiting substances in the medium. As long as the composition of the medium remains constant, the rate of growth does not vary. After more than 14 years of life *in vitro*, a strain of fibroblasts is growing at the same speed as during the first years of cultivation.

¹² Carrel, A., *J. Exp. Med.*, 1913, xvii, 14.

¹³ Carrel, A., *J. Exp. Med.*, 1923, xxxviii, 521.

and is left undisturbed in the coagulum, while the fluid medium consisting chiefly of embryonic juice is changed every 2 days, it grows at the optimum rate, and the curve expressing the increase in area is a parabola. After about 2 weeks, when the diameter of the colony has reached approximately 15 mm., the growth stops spontaneously. The tissue will resume its activity only after division and transplantation into another flask. While fibroblasts never cover a large area of the coagulum, macrophages often invade the entire medium in less than 3 weeks. The impossibility of a colony of fibroblasts growing beyond a certain size may be attributed to its peculiar architecture and to the solidarity of the cells. When two cells are united by their processes, and one is punctured with a needle and killed, the death of the other cell follows after a few seconds, as Chambers has shown.⁸ Although Lewis believes that there is no anatomical communication between cells united by their processes, it is obvious that they are in close relation.⁹ This condition is probably required for cell proliferation. Fischer found that cells isolated in a culture medium do not proliferate.¹⁰ In order for multiplication to occur, fibroblasts must be in close contact. This inherent property of forming a tissue may cause the limitation in size of the colonies. After the colony has reached a certain thickness, its central part probably becomes isolated from the food supply and degenerates, and toxic substances are set free which stop cell proliferation. We have often observed that the formation of a thick center in a colony is accompanied by marked changes in the appearance of the peripheral cells, which display a tendency to grow in scattered formation. Twice in the course of 14 years, a transformation of these scattered cells into macrophages occurred.

Fibroblasts have a special affinity for other fibroblasts, but not for epithelial cells. When a colony of pure epithelium is grown side by side with a colony of fibroblasts, the fibroblasts rapidly surround the epithelium, as was shown in an experiment by Ebeling and Fischer.¹¹

⁸ Chambers, R., in Cowdry, E. V., *General cytology*, Chicago, 1924, 242.

⁹ Lewis, W. H., and Lewis, M. R., in Cowdry, E. V., *General cytology*, Chicago, 1924, 393.

¹⁰ Fischer, A., *Tissue culture; studies in experimental morphology and general physiology of tissue cells in vitro*, Copenhagen, 1925, 155.

¹¹ Ebeling, A. H., and Fischer, A., *J. Exp. Med.*, 1922, xxxvi, 285.

boundaries of the anterior process through its open end. Continuous streaming of the cytoplasm in a straight line toward the free medium is a characteristic of the fibroblast. In exceptional instances, cells were observed moving backward. They almost never turned in a circle. Practically every cell in the peripheral area of a colony showed a very active outer pole and an inactive inner pole. The pictures emphasized the profound difference between this mode of locomotion and that of the macrophages. The latter cells are encircled by a thin undulating membrane which moves unceasingly and molds its folds on the surrounding bodies.

The motion of the protoplasmic structure was studied on pictures taken with a magnification of 300 to 400 diameters and at a rate of sixty exposures per minute. When the film was projected at the rate of ten exposures per second, the velocity of the motion was increased only ten times. It became possible to ascertain the relative displacements of the nucleus and of the cytoplasmic organs on preparations stained with neutral red, and on unstained preparations under dark-field illumination. When the motion of the cells was observed with a tenfold increase, the nucleus did not seem to undergo any displacement or change in form. On the contrary, the nucleoli modified their shapes continually, as already observed by Lewis.¹⁴ The fat globules, which were generally present in small number in the cells fed upon tissue juice, had no motion of their own. The larger they were, the more inert they appeared. The neutral red vesicles moved incessantly, and the amplitude of their oscillations was small. Nucleus, fat globules, and neutral red vesicles formed the main part of the body and followed the displacement of the cell as a whole, without their relative position being altered to any great extent. The more fat a fibroblast contained, the less agile it became. The small red granules could be divided into two classes according to their mode of locomotion, the stationary granules, and the wandering granules. Some of the stationary granules were located in the stained vesicles. Their characteristics have been minutely described by Renaut,¹ and later by Lewis.⁵ Some others were apparently free within the body or the processes. The tracings made of the successive positions they occu-

¹⁴ Lewis, W. H., *Anat. Rec.*, 1923, xxvi, 15.

While multiplying at their optimum rate, the fibroblasts store up some food material which is responsible for the growth energy they display when transplanted into a medium composed chiefly of Tyrode solution. During starvation, their residual energy permits the increase in area of the colony for a period varying from 6 to 8 days. The quantity of the reserves which the cells accumulate depends on the time they spend in a medium containing embryo juice and on the concentration of the juice in the medium. A maximum is rapidly reached beyond which no more food is stored up. The residual energy of the fibroblast appears to depend on its specific property of accumulating a certain amount of food material while being cultivated in a nutrient medium.

Mode of Locomotion of the Fibroblast and Movements of the Protoplasmic Structures.

The locomotion of the cells was studied on cinematographic films of pure cultures. The photographs were taken at a rate of three to six exposures per minute during 24 or 48 hours. When the films were projected at a rate of ten exposures per second, the velocity of the motion was increased from 100 to 200 times. As the magnification was only 70 diameters, a large area of the medium could be kept under observation. The cells progressed in straight lines. At a temperature of 39°C., their speed was uniform and reached approximately 33.3 μ per hour. Practically all the cells of a colony moved away from the center. Their activity was polarized, the distal end of the cells projecting its processes into the medium, while the proximal end merely followed. The front processes flowed through the medium, adhered to some of the structures, and kept the body of the cell stretched. If one of the processes lost its hold on the fibrin of the medium, the body suddenly jerked toward the processes that were still adherent to the clot. The protoplasm streamed through the front process, then the nucleus and the cytoplasmic structures of the body glided forward, dragging the rear process. Under dark-field illumination, the body and the processes were defined by a sharp line of beautiful geometrical design. At the end of the processes, which were always open, the boundary between cytoplasm and medium appeared very faint and irregular. The cytoplasm flowed between the lateral

stances contained in the juice of chick, mouse, guinea pig, and rabbit embryos.¹⁶ This characteristic distinguishes fibroblasts from macrophages which die when cultivated in pure embryo juice, and multiply in a medium composed exclusively of plasma.¹⁷ Both fibroblasts and epithelial cells possess the property of feeding upon embryo juice. But the effect of the juice is more marked on connective tissue than on epithelium. Fibroblasts also have the property of proliferating when fed upon Witte's peptone.

Fibroblasts and macrophages differ in a striking manner regarding their susceptibility to arsenous oxide. Arsenous oxide in concentrations varying from 1/50,000 to 1/3,000,000 was added to the medium of a 14 year old strain of fibroblasts, and the rate of growth of the colonies was measured. When the arsenic was present at 1/3,000,000 in the medium, the speed of proliferation decreased slightly. The growth completely stopped when the arsenous oxide reached a concentration of 1/800,000. This concentration of arsenic was entirely innocuous to macrophages, which were not killed by a concentration of 1/200,000. The susceptibility of fibroblasts to arsenous oxide renders it possible to obtain pure cultures of macrophages from mixed cultures by using media containing 1/300,000 arsenous oxide.

Relations between Protoplasmic Structures, Nature of Medium, and Rate of Growth of Colonies.

The colonies were composed of chicken fibroblasts belonging to a 14 year old strain, or to strains obtained more or less recently from the subcutaneous connective tissues of adult animals. They were cultivated in three different media, chiefly composed of serum or Tyrode solution or embryonic tissue juice, according to two techniques that have been previously described.¹⁸ The rate of growth of the colonies was measured by the ordinary methods.¹⁹ When the fibroblasts had multiplied for a few days and the rate of growth had been ascertained, they were transferred onto a cover-glass for examination. From 1 to

¹⁶ Carrel, A., and Ebeling, A. H., *J. Exp. Med.*, 1923, xxxviii, 499.

¹⁷ Carrel, A., and Ebeling, A. H., *J. Exp. Med.*, 1922, xxxvi, 365.

¹⁸ Carrel, A., *J. Exp. Med.*, 1923, xxxviii, 407.

¹⁹ Ebeling, A. H., *J. Exp. Med.*, 1921, xxxiv, 231.

pied in a number of exposures showed that they covered an oblong or circular area and that they moved inside an invisible vacuole or vesicle. The wandering granules could travel all over the cell from the central part of its body to the far end of the processes and back. Under dark-field illumination, they could not be mistaken for round mitochondria because, as already observed by Lewis,⁵ the minute mitochondria are not usually found in fibroblasts that show the small neutral red granules. With dark-field illumination, the unstained filamentous mitochondria were observed to move, as already described.⁴ The swift motion of the wandering granules and of the mitochondria indicated the location of free paths within the body. The wandering granules passed rapidly by the stationary granules, which moved back and forth in invisible vacuoles. They might also travel around the neutral red vesicles and the fat globules of the body. It is obvious that certain granules move freely around the bodies described by Renaut¹ and Evans² as normal segregation apparatus, and by Lewis⁵ as degeneration vacuoles. The vacuoles cannot be considered as free spaces, full of fluid, in a jelly-like cytoplasm. They are evidently vesicles, empty or containing a granule. As the path of the wandering granules is irregular and broken, it is probable that the cytoplasm has a sponge-like structure. It is not a gel, a fact shown by the rate of motion of the filamentous mitochondria, as well as by that of the minute red granules. The body of the fibroblast appears to be composed mainly of small vesicles, that do or do not stain with neutral red, and between which fat globules may accumulate. In the perivesicular fluid wander the small neutral red granules and the filamentous mitochondria. The conception of the cytoplasm as a mass of jelly containing some vacuoles full of fluid should be discarded as inaccurate.

Food Requirements of the Fibroblast. Its Susceptibility to Arsenous Oxide.

Fibroblasts do not feed on plasma, egg albumin, egg yolk, amino acids, or broth.¹⁵ They synthesize protoplasm exclusively from sub-

¹⁵ Carrel, A., and Ebeling, A. H., *J. Exp. Med.*, 1921, xxxiv, 317, 599; 1923, xxxviii, 487.

Experiment 2.—Culture 3338-C, Feb. 24. Feb. 26, stained with neutral red and Janus green; camera lucida drawings. The cells which were close to the original fragment were 120μ in length, and the nucleus was 15μ ; the cells contained 20 small neutral red vesicles about 0.5μ in diameter; 4 or 5 wandering red granules; about 15 small fat globules; 35 mitochondria averaging 2.5μ in length (Fig. 3). Some of the cells which had wandered out into the medium a short distance from the original fragment of tissue showed about 37 neutral red vesicles 0.75μ in diameter; 4 or 5 wandering stained granules; 35 mitochondria, 2.5 to 3μ in length (Fig. 4).

Experiment 3.—Culture 3387-C, Mar. 2. Mar. 4, camera lucida drawings. 20 to 25 small neutral red vesicles, 0.5μ in diameter; 6 to 8 wandering granules; 50 to 60 mitochondria, averaging 1.75μ in length; some cells show a few fat globules; length of nucleus averages 15μ ; length of cell, 85 to 95μ (Figs. 5 and 6).

Experiment 4.—Culture 2876-C, Jan. 5. Jan. 8, first passage, Culture 2901-C. Jan. 9, camera lucida drawings; length of cell, 75μ ; length of nucleus, 15μ ; 25 vesicles, 1μ in diameter; about 10 fat globules; a few wandering neutral red granules less than 1μ in diameter (Fig. 7).

Experiment 5.—Culture 2884-C, Jan. 6. Jan. 9, first passage, Culture 2907-C. Jan. 11, second passage, Culture 2917-C. Jan. 12, camera lucida drawings. Length of cell, 95μ ; length of nucleus, 19μ ; 30 to 40 red vesicles, 1μ in diameter; a few very small wandering granules, less than 0.5μ in diameter; about 10 small fat granules (Fig. 8).

Subcutaneous Connective Tissue from a Tumor Chicken, Cultivated in Equal Volumes of Plasma and Embryonic Tissue Juice.

Experiment 6.—Culture 3206-C, Feb. 8. Feb. 11, culture stained with cresyl blue; camera lucida drawings. Length of cell, 95μ ; length of nucleus, 14μ ; a few minute fat granules; 40 to 60 faintly stained vesicles, 0.5 to 1μ in diameter, generally containing 1 small granule, darkly stained; 35 to 40 mitochondria, 3 to 10μ long; a few wandering granules less than 0.5μ in diameter (Fig. 9).

Experiment 7.—Culture 2842-C, Dec. 23, 1925. Dec. 31, first passage, Culture 2856-C. Jan. 2, 1926, second passage, Culture 2859-C. Jan. 4, third passage, Culture 2867-C. Jan. 5, camera lucida drawings. Length of cell, 140μ ; length of nucleus, 19μ ; 30 to 65 vesicles, 2μ in diameter; about 8 or 10 small wandering granules, 0.5μ in diameter; about 15 small fat globules (Fig. 10). Jan. 7, fourth passage, Culture 2890-C. Jan. 9, camera lucida drawings. Length of cell, 80μ ; length of nucleus, 19μ ; 40 to 50 red vesicles; 7 to 10 small fat globules; a few small red granules (Fig. 11).

Subcutaneous Connective Tissue from an Adult Chicken, Cultivated in Normal Plasma and Embryonic Juice.

Experiment 8.—Culture 2932-C, Jan. 12. Jan. 22, the tissues were placed in plasma containing a trace of tissue juice until Jan. 29. Jan. 30, Culture 3104-C,

48 hours after the slides were prepared, the tissues were stained with 1/20,000 Janus green and 1/20,000 neutral red, and camera lucida drawings were made of the cells at a magnification of 1600 or 3200 diameters.

1. Conditions of Neutral Red Vesicles and Mitochondria in a 14 Year Old Strain of Fibroblasts.—The strain has been kept for 14 years in equal volumes of chicken plasma and embryonic tissue juice. The rate of growth is such that the colonies double in volume every 48 hours. Tissue fragments in a condition of optimum activity were stained with neutral red and Janus green, and camera lucida drawings made.

Generally, the length of the cells varied from 100 to 140 μ , and that of the nucleus from 18 to 22 μ . Neutral red vesicles and small fat globules were located in unequal quantities in the cytoplasm at both ends of the nucleus. The vesicles were from 1 to 1.5 μ in diameter. Their number varied approximately from 50 to 100. They often contained a small red granule. There were also minute wandering granules in the body of the cell and in its processes. Their diameter was less than 1 μ . The vesicles accumulated usually in the anterior part of the body, probably around the centriole, while the mitochondria were located around the nucleus, in the processes, and between the vesicles. They were long, filamentous, and of even caliber. The dumb-bell, vesicular, and other abnormal forms described by Lewis and Lewis⁴ were not present (Fig. 1).

2. Neutral Red Vesicles, and Mitochondria in Fibroblasts from Adult Connective Tissue.—Fragments of subcutaneous connective tissue were extirpated from young adult chickens. They were cultivated in a medium composed of equal parts of plasma and embryonic tissue juice. Every 2 days, the tissues were removed from the containers, washed in Tyrode solution, and transferred to a fresh medium. From 24 hours to 2 weeks after the extirpation of the tissues, the cultures were stained with neutral red and Janus green, separately or in combination, and camera lucida drawings made of the cells.

Subcutaneous Connective Tissue from an Adult Chicken, Cultivated in Equal Volumes of Plasma and Embryonic Juice.

Experiment 1.—Culture 2956-C, Jan. 14, 1926. Jan. 15, camera lucida drawings. Length of nucleus, 18 μ ; length of cells, 90 μ ; no neutral red vesicles; 2 to 5 small red granules wandering through the processes; 51 fat globules (Fig. 2).

and tissue juice are placed in a coagulum composed almost exclusively of plasma, the rate of growth immediately decreases and proliferation ultimately stops. In the present experiments, the colonies were divided into halves, one being placed in plasma with a trace of tissue juice, and the other in equal parts of plasma and embryo juice. After a few days cultivation, the relations between the rate of growth and the morphological changes could be easily studied.

The fibroblasts were obtained from the subcutaneous connective tissue of adult chickens and prepared from the experiments by being cultivated for a few weeks in plasma and embryonic tissue juice. After their rate of growth and appearance had become analogous to those of the 14 year old strain, the tissues were divided. One half was cultivated in a medium composed of 1 volume of plasma and 1 volume of Tyrode solution containing a trace of embryonic tissue juice, and the other half in equal parts of plasma and embryonic tissue juice. The relative increase of both colonies was measured by a technique previously described.¹⁹ Cinematograph pictures have shown that practically every fibroblast at the edge of a colony moves in straight lines from the center of the colony toward the free medium and that the mechanical activity of the front process is far greater than that of the rear process, which appears to move along, dragged by the body. As the anterior pole of the cells is in a more active condition than the posterior one, it may be assumed that the organs which congregate at the root of the forward processes possess some function related to heightened metabolism. When a marked difference was observed in the rates of growth of the experiment and control tissues, the cells were stained with Janus green and neutral red, and camera lucida drawings made. Ten experiments were performed.

Experiment 1.—30 day old strain of adult connective tissue, Culture 3302-C, Feb. 17. A colony was divided into two parts, one being cultivated in plasma, and the other in 1 volume plasma and 1 volume embryonic juice. Feb. 19, first passage in fresh media; Feb. 20, second passage in fresh media; measurement of the rate of growth and camera lucida drawings.

1. Fibroblasts cultivated in plasma. Rate of growth, 1.1; length of average cell, 120μ ; length of nucleus, 18 to 19μ ; 20 neutral red vesicles, 0.75μ in diameter, of which more than half were localized in anterior part of the body; 50 mitochondria, 2 to 5μ in length; many fat globules, 0.25 to 1.25μ in diameter in anterior and posterior portions of cell body; about 25 deeply stained neutral red wandering granules, 0.25μ in diameter.

camera lucida drawings. Length of cell, 85μ ; length of nucleus, 15μ ; 1 to 5 small vesicles, less than 0.5μ in diameter; 10 to 15 elongated vesicles, stained with neutral red, 1.5 to 2μ long; no fat globules; about 10 filamentous mitochondria.

Subcutaneous Connective Tissue from an Adult Chicken, Cultivated in Plasma Containing a Trace of Embryonic Juice.

Experiment 9.—Culture 2892-C, Jan. 7. Jan. 11, first passage in same medium, Culture 2920-C. Jan. 13, camera lucida drawings. Length of cell, 85μ ; length of nucleus, 18μ ; 7 to 8 neutral red vesicles, 0.25 to 0.5μ in diameter; 4 to 6 neutral red wandering granules, about 0.25μ in diameter; 0 to 8 small fat globules, 0.5μ in diameter (Fig. 12).

In these experiments, a comparative study was made of the number and size of the neutral red vesicles in fibroblasts maintained for 14 years *in vitro* in a condition of optimum activity, and in adult fibroblasts cultivated for periods varying from 24 hours to 13 days, in embryonic tissue juice or in Tyrode solution. The fibroblasts which had proliferated with great activity for 14 years in equal parts of plasma and embryo juice always contained many neutral red vesicles (Fig. 1). Adult connective tissue, on the contrary, cultivated in a similar medium for 24 hours grew very slowly, and the cells contained none or very few vesicles, as shown in Fig. 2. The neutral red vesicles became more numerous and cell proliferation more rapid after 48 hours cultivation in embryonic tissue juice (Figs. 3 to 6). The phenomenon was more evident after 72 hours cultivation (Fig. 7), and after a week (Figs. 8 and 9). When the cells derived from adult connective tissue had been cultivated for a longer time in embryonic tissue juice (Figs. 10 and 11), the number of vesicles was about as great as in the 14 year old strain. However, if embryonic tissue juice was left out of the medium and replaced by Tyrode solution, the activity of the adult connective tissue cells did not increase, and the number of vesicles remained small (Fig. 12).

3. *Changes in the Structure of Fibroblasts and the Rate of Growth, Brought About by Variations in the Composition of the Medium.*—An attempt was made to ascertain in a more precise manner how the protoplasmic organs are modified by metabolic changes. It is well known that the activity of fibroblasts is affected by the transfer from a nutrient to a non-nutrient medium. If colonies cultivated in plasma

1. Fibroblasts in plasma. Rate of growth, 1.2; length of average cell, 90μ ; length of nucleus, 16μ ; 22 neutral red vesicles, 0.25 to 0.75μ in diameter, located in anterior part of the body; 26 wandering neutral red granules; 75 fat globules located in posterior part; 90 mitochondria, 5 to 10μ in length.

2. Fibroblasts in plasma and embryonic juice. Rate of growth, 4; length of average cell, 80μ ; length of nucleus, 16μ ; 135 neutral red vesicles, 0.5 to 1μ in diameter, located in anterior and lateral parts of body at root of large side process; 14 small fat globules in posterior part; 60 mitochondria, 5 to 8μ in length.

Experiment 5.—19 day old strain of adult fibroblasts, Culture 3531-C, Mar. 15. Fragment divided into two parts, one being cultivated in plasma, and the other in equal volumes of plasma and embryonic juice. Mar. 18, first passage into same media; Mar. 19, camera lucida drawings.

1. Fibroblasts in plasma. Rate of growth, 1.5; length of average cell, 105μ ; length of nucleus, 19μ ; 16 neutral red vesicles, 0.25 to 0.50μ in diameter, located in anterior part of the cell body; 36 filamentous mitochondria, 5 to 15μ in length; 47 small fat globules.

2. Fibroblasts in plasma and embryonic juice. Rate of growth, 10.9; length of average cell, 110μ ; length of nucleus, 19.5μ ; 140 neutral red vesicles, 0.25 to 1.5μ in diameter, located in anterior part of the cell body lateral to the nucleus; disposition of most of the vesicles around centriole; 40 mitochondria, 3 to 6μ in length; 24 fat globules; a group of larger ones posterior to the nucleus.

Experiment 6.—14 year old strain of fibroblasts, Culture 3068-A, Feb. 15. A fragment was divided into two parts, which were cultivated in two flasks in the ordinary medium. Feb. 16, both cultures were washed and patched. Feb. 17, both cultures were washed for 10 minutes with Tyrode solution, then 0.5 serum and 0.5 Tyrode solution were introduced into Flask A, and 1 cc. of pure embryo extract into Flask B. Feb. 19, both cultures were washed and the same media introduced. Feb. 20, growth had stopped in Flask B, while it was still rapid in Flask A. Measurement of the area covered by the fibroblasts, transfer of the tissues to cover-glasses, and camera lucida drawings.

1. Fibroblasts cultivated in Tyrode solution. Growth stopped; length of average cell, 90μ ; length of nucleus, 13 to 15μ ; 8 neutral red vesicles, 0.25 to 0.5μ in diameter; 10 wandering neutral red granules; 50 mitochondria, 1 to 2μ in length; 54 fat globules around nucleus and posterior to it.

2. Fibroblasts cultivated in embryonic juice. Rate of growth, 3; length of average cell, 110μ ; length of nucleus, 18μ ; 42 neutral red vesicles, 0.25 to 1.25μ in diameter; 14 wandering neutral red granules; 50 mitochondria, 2 to 7μ in length; 46 fat globules around nucleus and posterior to it.

Experiment 7.—36 day old strain of adult connective tissue, Culture 3348-C. A fragment was divided into two parts, one being cultivated in 3 volumes Tyrode solution and 1 volume plasma, and the other in 3 volumes embryonic juice and 1 volume plasma. Feb. 26, camera lucida drawings (Figs. 19 and 20).

1. Fibroblasts in Tyrode solution. Rate of growth, 0.77; length of average cell, 120μ ; length of nucleus, 17μ ; 14 neutral red vesicles, 1μ in diameter; 15 wandering neutral red granules; 100 mitochondria, 1 to 2μ in length; no fat globules.

2. Fibroblasts cultivated in plasma and embryonic juice. Rate of growth, 2.3; length of average cell, 130μ ; length of nucleus, 21μ ; 70 neutral red vesicles, 0.5 to 1μ in diameter in anterior and lateral parts of the body and at root of a large lateral process. A few vesicles contained deeply stained neutral red granules; 60 mitochondria, thin and regular, 3 to 7μ long; 21 fat globules, 0.5μ in diameter; 22 wandering neutral red granules.

Experiment 2.—28 day old strain of adult connective tissue, Culture 3314-C, Feb. 17. Division of a colony into two parts, one being cultivated in plasma, and the other in equal parts of plasma and embryonic juice. Feb. 19, first passage in fresh media; Feb. 21, second passage; Feb. 23, third passage; Feb. 24, camera lucida drawings (Figs. 13 and 14).

1. Fibroblasts in plasma. Rate of growth, 4.6; length of average cell, 110μ ; length of nucleus, 15μ ; 100 neutral red vesicles, 0.25 to 1μ in diameter, distributed more abundantly in anterior part of the body; no granules in the vesicles; 6 wandering neutral red granules, less than 0.25μ in diameter; 80 mitochondria, 1 to 3μ in length; 20 fat globules.

2. Fibroblasts in plasma and embryonic juice. Rate of growth, 11.0; length of average cell, 100μ ; length of nucleus, 16 to 17μ ; two-thirds of the mass of neutral red vesicles, about 140 in number, in anterior part of the body, 0.5 to 1.25μ in diameter. 25 fat globules scattered among vesicles; 17 wandering neutral red granules, about 0.25μ in diameter; 70 mitochondria, slender and regular, 6 to 7μ in length.

Experiment 3.—47 day old strain of adult connective tissue, Culture 3331-C, Feb. 17. Division of a colony into two parts, one being cultivated in plasma, and the other in plasma and embryonic tissue juice. Feb. 19, first passage in the same media; Feb. 21, second passage in the same media; Feb. 23, third passage in same media; Feb. 24, camera lucida drawings (Figs. 15 and 16).

1. Fibroblasts cultivated in plasma. Rate of growth, 0.97; length of average cell, 100μ ; length of nucleus, 18μ ; 50 neutral red vesicles, 0.5 to 0.75μ in diameter, 5 containing small neutral red granules; many small vesicles, less than 0.5μ in diameter; more vesicles in anterior part of the body than in posterior; 80 mitochondria, 1 to 3μ in length; 3 or 4 wandering neutral red granules; no fat globules.

2. Fibroblasts in embryonic juice and plasma. Rate of growth, 2.82; length of average cell, 130μ ; length of nucleus, 20μ ; 195 vesicles, 0.25 to 1μ in diameter, more being located in anterior part of cells than in posterior; many very small neutral red vesicles scattered throughout cytoplasm; about 35 wandering neutral red granules in the cell processes; 90 mitochondria, 3 to 9μ in length; 35 small fat globules.

Experiment 4.—41 day old strain of adult fibroblasts, Culture 3253-C, Feb. 10. Fragment divided into two parts, one being cultivated in plasma, and the other in plasma and embryonic tissue juice. Feb. 12, first passage in same media; Feb. 13, second passage in same media; Feb. 15, third passage in same media; Feb. 16, camera lucida drawings (Figs. 17 and 18).

2. Fibroblasts cultivated in embryo juice. Rate of growth, 2.5; length of cell, 110μ ; length of nucleus, 15μ ; 100 neutral red vesicles, 0.25 to 1.5μ in diameter; 13 wandering red granules; 55 mitochondria, 2 to 12μ in length; 45 small fat globules.

In these ten experiments, fibroblasts in a known condition of activity and showing long filamentous mitochondria and a number of small neutral red vesicles (Figs. 14, 16, 18, 20, 22, and 24) were cultivated in media deprived of embryonic juice and composed chiefly of serum or Tyrode solution. Their rate of proliferation immediately decreased and at the same time their structure underwent marked changes. The cytoplasmic organs of the cells living in plasma alone assumed the appearance of those of resting fibroblasts (Figs. 13, 15, and 17). The granules disappeared from the vesicles. The neutral red vesicles were reduced to one-half or one-third of their normal number. At the same time, the size of the nucleus decreased, and the mitochondria became shorter. No modification took place in the controls, which were maintained in embryonic juice. The simultaneous variations of the rate of growth and of the protoplasmic organs are summarized in Table I. When the colonies were cultivated in Tyrode solution, the changes became more apparent (Figs. 19, 21, and 23). The decrease in the size of the nucleus was greater. For instance, in Experiment 8, where cell proliferation had completely stopped, the nucleus was only 11μ in length while it was 22μ in the control (Figs. 21 and 22). The mitochondria were not greatly modified in number but, instead of being filaments 5 to 6μ in length, they had become very short rods or dots. The fat globules were much more abundant. The neutral red vesicles were reduced to one-tenth of the number present in the control. They were smaller and contained no granules (Table I). The results of both series of experiments may be summarized as follows:

The significance of the neutral red vesicles observed in normal cells has been investigated by studying fibroblasts artificially placed in definite states of activity. The lowest metabolic condition was obtained by cultivating fragments of subcutaneous connective tissue, removed from an adult chicken, in a medium composed almost exclusively of plasma. The first cells which slowly migrated from the fragment were almost in a resting condition. After a few days cultivation in plasma and embryonic juice, their activity increased, as shown by measurement of their rate of growth which finally became equal to

2. Fibroblasts in embryonic juice. Rate of growth, 3.28; length of average cell, 140μ ; length of nucleus, 22μ ; 195 neutral red vesicles, 0.5 to 1μ in diameter; 10 vesicles with neutral red granules; 63 mitochondria, 2 to 9μ in length; no fat globules.

Experiment 8.—14 year old strain of fibroblasts, Culture 3303-C, Feb. 15. Two flasks were prepared, each containing four fragments of the old strain, cultivated in ordinary medium. Feb. 16, both cultures washed; 1 cc. of Tyrode solution was introduced into Flask A, and 1 cc. of embryonic juice into Flask B. Feb. 17, both cultures washed in Tyrode solution and given fresh media; measurement of relative increase; Feb. 18, same treatment; Feb. 19, measurement of increase, transfer of cultures to glass slides, and camera lucida drawings (Figs. 21 and 22).

1. Fibroblasts in Tyrode solution. Rate of growth, 1; length of average cell, 100μ ; length of nucleus, 11μ ; 49 neutral red vesicles, 0.25 to 0.5μ in diameter; 5 wandering neutral red granules; 60 mitochondria, 0.25 to 2μ in length; 50 fat globules.

2. Fibroblasts in embryonic juice. Rate of growth, 7; length of average cell, 120μ ; length of nucleus, 22μ ; 190 neutral red vesicles, 0.25 to 1μ in diameter; 15 wandering neutral red granules; 50 mitochondria, 1 to 5μ in length; 18 fat globules.

Experiment 9.—Culture 3502-C, Mar. 1. A fragment of chick embryo heart was divided into two parts, one being cultivated in 1 volume plasma and 3 volumes Tyrode solution containing a trace of embryonic juice, and the other in 1 volume plasma and 3 volumes embryonic juice. In the course of 16 days, both fragments were transferred six times into fresh media. Mar. 17, measurement of rate of growth, and camera lucida drawings.

1. Fibroblasts cultivated in Tyrode solution. Rate of growth, 0.03; length of cell, 90μ ; length of nucleus, 11μ ; 5 neutral red vesicles, 0.25μ in diameter; 2 wandering red granules; 20 mitochondria, 1 to 2μ in length; 95 small fat globules around the nucleus.

2. Fibroblasts cultivated in embryonic juice. Rate of growth, 3.0; length of cell, 120μ ; length of nucleus, 18μ ; 195 neutral red vesicles, 1 to 2μ in diameter; 13 wandering red granules; 50 mitochondria, 3 to 6μ in length; 45 very small fat globules.

Experiment 10.—Culture 3623-C, Feb. 21. 8 day old strain of fibroblasts. Mar. 1, fragment was divided into two parts, one being cultivated in 1 volume plasma and 3 volumes Tyrode solution containing a trace of embryonic juice, and the other in 1 volume plasma and 3 volumes embryonic juice. In the course of 29 days, both fragments were transferred fifteen times into fresh media. Mar. 30, measurement of rate of growth, and camera lucida drawings (Figs. 23 and 24).

1. Fibroblasts cultivated in Tyrode solution. Rate of growth, 0.01; length of cell, 75μ ; length of nucleus, 11μ ; 11 neutral red vesicles, 0.25μ in diameter; 5 wandering red granules; 39 mitochondria, 2 to 5μ in length; no fat globules.

that of a pure strain of fibroblasts cultivated for 14 years in plasma and embryonic juice. The effects of decreased metabolism on protoplasmic structures were observed in a precise manner by depriving fibroblasts, which had reached their optimum rate of growth, of embryonic tissue juice and cultivating them in Tyrode solution and also in serum or plasma. The relations between the metabolic conditions as expressed by the rate of proliferation and the cell structure immediately became clear.

At the beginning of their life *in vitro*, the adult fibroblasts, and embryonic fibroblasts cultivated in plasma or in Tyrode solution until their activity had decreased or stopped, presented some common characteristics. Their cytoplasm showed very few small neutral red vesicles. These vesicles did not contain any granules. There were no fat globules in the body of the cell, and the mitochondria consisted of short filaments. Simultaneously with the increase of the rate of growth obtained by cultivation in embryonic juice, the neutral red vesicles became more numerous and the filamentous mitochondria longer. Small red bodies appeared within the vesicles, and a number of wandering granules moved through the process and the body. When the cells had reached their highest activity, the number of the vesicles became maximum, as well as the length of the mitochondria. The phenomenon was reversible. If colonies of the 14 year old strain of fibroblasts were placed in serum or in Tyrode solution, the rate of growth decreased and at the same time the vesicles lost their granules and were reduced in number, while the mitochondria became much shorter. The neutral red vesicles congregated in the anterior part of the body, that is, at the active pole. If a lateral process developed and became more active, the vesicles shifted to the root of this process. In either case, they were probably grouped around the centriole, as observed by Lewis.²⁰ But in normal fibroblasts, the centriole generally remains invisible. It was obvious that the number and size of the vesicles were related to metabolism and not to the duration of cultivation. Fibroblasts obtained from adult connective tissue and from the 14 year old strain, and cultivated in embryo juice, assumed the same appearance as soon as their respective rates of growth had become

²⁰ Lewis, W. H., *Anat. Rec.*, 1919, xvi, 155.

TABLE I.
Relation between the Rate of Growth and the Number and Size of Cell Structures.

Experiment No.	Culture No.	Fig. No.		Rate of growth.		Length of cell.		Length of nucleus.		No. of neutral red vesicles.		Diameter of neutral red vesicles.		No. of mitochondria.		Length of mitochondria.		No. of fat globules.		No. of wandering neutral red granules.	
		Plasma.	Tissue juice.	Plasma.	Tissue juice.	Plasma.	Tissue juice.	Plasma.	Tissue juice.	Plasma.	Tissue juice.	Plasma.	Tissue juice.	Plasma.	Tissue juice.	Plasma.	Tissue juice.	Plasma.	Tissue juice.	Plasma.	Tissue juice.
1	3302-C			1.1	2.3	120	130	18-19	21	20	70	0.75	0.5-1	50	62	2-5	3-7	60	21	25	22
2	3314-C	13	14	4.6	11.0	110	110	15	15-17	100	140	1	1	80	70	1-3	6-7	20	25	6	17
3	3331-C	15	16	0.97	2.82	100	130	18	20	50	195	0.5-0.75	0.25-1	80	90	1-3	3-9	0	35	3-4	35
4	3253-C	17	18	1.2	4.0	90	80	16	16	22	135	0.25-0.75	0.5-1	90	60	5-10	5-8	75	14	26	13
5	3531-C			1.5	10.9	105	110	19	19.5	16	140	0.25-0.5	0.25-1.5	36	40	5-15	3-6	47	24	5	9
		Tyrosine.												Tyrosine.				Tyrosine.			
6	3068-A			0.0	3.0	90	110	13-15	18	8	42	0.25-0.5	0.25-1	50	50	1-2	2-7	54	46	10	14
7	3348-C	19	20	0.77	3.28	120	140	17	22	14	195	1	0.5-1	100	63	1-2	2-9	0	0	15	35
8	3303-C	21	22	1.0	7.0	100	120	11	22	18	190	0.25-0.5	0.25-1	60	50	0.2-2	1-5	50	18	5	15
9	3502-C			0.03	3.0	90	120	11	18	5	195	0.25	1-2	20	50	1-2	3-6	95	45	2	13
10	3623-C	23	24	0.01	2.5	75	110	11	15	11	100	0.25	0.25-1.5	39	55	2-5	2-12	0	45	5	13

the cell within the organism. It became obvious that fibroblasts must be in a resting condition within the adult animal on account of their inherent property of requiring for multiplication substances which are not present in lymph or blood serum. It is also evident that they are capable of resuming their embryonic activity, even in extreme old age, during the process of wound healing, or of organ sclerosis, because they have the power of feeding on substances set free by leucocytes or by epithelial cells, as happens when adult connective tissue is cultivated in embryonic juice. These and other working hypotheses may be constructed on the knowledge of the biological properties of the fibroblast, in order to explain, partly at least, the mechanism of the complex phenomena in which connective tissue cells participate.

The study of pure strains of fibroblasts brought into definite states of activity by cultivation in various media, under such conditions that they do not degenerate, has led to a correlation of certain protoplasmic structures and nutritional conditions. Neutral red vesicles and their granules are evidently connected with cell metabolism. The vesicles are more numerous while the colonies develop rapidly; they lose their granules and decrease in number if proliferation becomes slower or stops after the colonies have been deprived of food. They appear to be organs of great importance, the development of which is intimately bound up with the physiological state of the cell. Evidently they elaborate substances which are either used by the cells in their rapid proliferation, or accumulated as reserves, and possibly as material for exogenous secretion. Whether stored up foodstuff, or secretory material, these substances are the result of protoplasmic activity, and are manufactured within vesicles which must be considered as the normal organs of the active fibroblast. These vesicles differ markedly from the vacuoles that develop around bacteria and foreign bodies,^{1,21} and from the degeneration vacuoles described by Lewis.⁵ Neutral red is not a specific stain. It colors normal and degenerating structures in the same manner. The presence in the connective tissue cells of different types of vacuoles was more clearly understood by the earlier anatomists than by the modern ones. The distinction between the secretory vacuoles and those that develop around a foreign body was

²¹ Prigosen, R. E., *Bull. Johns Hopkins Hosp.*, 1921, xxxii, 206.

similar. This fact shows that the morphology of a fibroblast does not depend on its origin, adult or embryonic, but on the presence and the concentration of certain substances in the pericellular fluid. The length of the cell, the size of the nucleus, the number, size, and content of the neutral red vesicles, the number of the wandering red granules, and the size of the mitochondria vary with the rate of growth of the colony and the composition of the medium (Table I). Then the structure of the cells can be modified at will, and the appearance that they will assume when living in media of definite composition may be accurately predicted. It has become possible to ascertain the physiological state of a fibroblast from its anatomical characteristics. The appearance of the cytoplasmic organs indicates whether a fibroblast is at rest, in a condition of moderate or optimum activity, or in a starving condition. The active pole of the cell and the direction of its migration can also be detected by the location of the neutral red vesicles.

DISCUSSION.

The determination of the specific physiological properties of the fibroblast is of evident importance, as the individuality of a cell depends on its functions far more than on its staining reactions. The morphological analysis of a protoplasmic structure merely leads to its identification. When a bacterium is only characterized by its shape and mode of reaction toward dyes, our knowledge of it is of an unsatisfactory nature, and must be completed by the study of its properties in pure cultures, such as its action on the medium, the food it requires for multiplication, the poisons it secretes, its susceptibility to various chemical substances, etc. The same is true of tissue cells. The most minute anatomical descriptions are without great interest as long as morphology is not correlated with function, and as the essential properties of the cell remain unknown. Through the foregoing experiments, the fibroblast has become characterized not only by the structure of its nucleus and of its mitochondria and neutral red vacuoles, but also by the appearance of its colonies, its action on the medium, its relation to other cells, its rate of proliferation under given conditions, its mode of locomotion, the movements of its cytoplasmic organs, the food it requires for multiplication, etc. This new knowledge has made it possible to understand and to foresee the behavior of

undergo some variations which are definitely related to those of cell metabolism. In the more active colonies, they are long filamentous organs from 5 to 10 μ in length, which present the morphological characteristics minutely described by Lewis and Lewis.⁴ The irregular and abnormal forms, which they also mentioned, were rarely observed. It is probable that they are more often present in cells that begin to degenerate. The normal mitochondria were seen to become shorter and slightly thicker when the rate of growth of the colonies decreased. They broke down into dots in cells starved in Tyrode solution. If the activity of the cells was increased by cultivation in embryonic juice, the mitochondria again became longer. Their number did not vary greatly. Their mode of distribution through the body and the active and less active processes remained approximately constant. They did not congregate in the region of the cell which displayed the greatest mechanical activity, as neutral red vesicles do. Mitochondria are certainly endowed with a function more fundamental to the life of the cell than the neutral red vesicles, because they always remain present in resting cells, when the neutral red vesicles have almost completely disappeared. So far, it has generally been believed, as expressed by Cowdry,²² that mitochondria possess a fundamental rôle in cell activity. But the nature of this rôle is not as yet clearly understood. Lewis⁴ thought that the quantity of mitochondria differs so widely that it is impossible to connect it with any definite function. He believed that these great variations depended on the metabolism of the individual cells. The truth of this hypothesis was demonstrated by our experiments. All the fibroblasts which compose the outer edge of a growing colony are in identical metabolic condition. The appearance of the cells becomes uniform. There is but little variation in the size and quantity of the mitochondria in the fibroblasts of a given culture. However, when the activity of the whole colony is affected by a change in the medium, the mitochondria of every visible cell undergo parallel modifications.

From the foregoing experiments, it may be concluded that:

1. The fibroblast is characterized not only by its morphology and

²² Cowdry, E. V., *Carnegie Institution of Washington, Pub. No. 271, Contributions to Embryology*, 1919, viii, 39.

made long ago by Renaut in his masterly investigations of the connective tissue cells.¹ The degeneration vacuoles remained unknown to him because his observations were made exclusively on tissues freshly extirpated from the animal. The degeneration vacuoles are a modern product of defective techniques and develop in cells which are erroneously believed to be in a condition of cultivation. The neutral red vesicles observed by Renaut¹ in the fibroblast and the macrophage were identical with those described as the normal segregation apparatus by Evans and Scott² in their excellent study of the connective tissue cells, and with those observed by us at every stage of their development *in vitro*.

In the early observations by Renaut, the vesicles assumed various characteristics according to the age of the animals from which the tissues were obtained. Very few were observed in the normal connective tissue of adult animals. But in fetuses or very young animals, every fibroblast presented some red vacuoles containing a segregation granule. The number of the stained cells was found to decrease with advancing age and in the old animal they almost completely disappeared. Renaut concluded that the presence of the neutral red vesicles is a characteristic of the young fixed connective tissue cell.³ He corroborated these findings by a study of the tendons of the tail of the rat, mouse, rabbit, and cat. In young animals, every fibroblast was seen to contain neutral red vesicles, while in older animals, none of the cells stained red. The presence of the vesicles was evidently related to a function of the fibroblast which persisted as long as the tendon fibers were not definitely organized. Young cells and cells in a condition of secretory activity appeared to be endowed with the property of elaborating segregation granules within the fluid of vesicles. Renaut gave to those cells the name of *rhagiocrin*, from *ράγιον*, grape, because granules growing in a vesicle resemble the seeds of a grape. The segregation apparatus of the fibroblast and the macrophage was considered by him as a common characteristic of both cells, the macrophage having an apparatus very much more developed than the fibroblast.

The results of our experiments are in agreement with this early conception of the functions of the neutral red vacuoles. It is certain that they represent an organ which characterizes cells in a condition of metabolic activity, and that their development may be increased or decreased at will by the substances which modify the rate of growth. The normal segregation apparatus of the fibroblast differs from both degeneration vacuoles and vacuoles formed around foreign bodies.

The length and number of the mitochondria have been found to

PLATE 7.

FIG. 13. Culture 3314-C. Fibroblasts in plasma. Rate of growth, 4.6.

FIG. 14. Culture 3314-C. Fibroblasts in plasma and embryonic juice. Rate of growth, 11.0.

FIG. 15. Culture 3331-C. Fibroblasts in plasma. Rate of growth, 0.97.

FIG. 16. Culture 3331-C. Fibroblasts in plasma and embryonic juice. Rate of growth, 2.82.

FIG. 17. Culture 3253-C. Fibroblasts in plasma. Rate of growth, 1.2.

FIG. 18. Culture 3253-C. Fibroblasts in plasma and embryonic juice. Rate of growth, 4.0.

FIG. 19. Culture 3348-C. Fibroblasts in Tyrode solution. Rate of growth, 0.77.

FIG. 20. Culture 3348-C. Fibroblasts in embryonic juice. Rate of growth, 3.28.

FIG. 21. Culture 3303-C. Fibroblasts in Tyrode solution, from same experiment. One stained with neutral red and the other with Janus green. Rate of growth, 1.0.

FIG. 22. Culture 3303-C. Fibroblasts in embryonic juice, one stained with neutral red and the other with Janus green. Rate of growth, 7.0.

FIG. 23. Culture 3623-C. Fibroblasts in Tyrode solution. Rate of growth, 0.01.

FIG. 24. Culture 3623-C. Fibroblasts in embryonic juice. Rate of growth, 2.5.

staining reaction, but also by a number of physiological properties, which become apparent in the pure cultures of the cell.

2. There is a definite relation between the size, the number, and the form of certain cytoplasmic structures and the metabolic conditions of the fibroblast.

EXPLANATION OF PLATES.

PLATE 6.

FIG. 1. Fibroblasts cultivated for 14 years in equal parts of plasma and embryonic juice. Camera lucida drawing made 24 hours after passage into fresh medium.

FIG. 2. Fibroblasts from subcutaneous connective tissue of young adult chicken, cultivated in equal volumes of plasma and embryonic juice. Camera lucida drawing made after 24 hours incubation.

FIGS. 3 and 4. Fibroblasts from subcutaneous connective tissue of an adult chicken, cultivated for 48 hours in equal volumes of plasma and embryonic juice. Camera lucida drawing made after 48 hours incubation.

FIGS. 5 and 6. Fibroblasts from subcutaneous connective tissue of an adult chicken, cultivated for 48 hours in equal volumes of plasma and embryonic juice. Camera lucida drawing made after 48 hours incubation.

FIG. 7. Fibroblasts from subcutaneous connective tissue of an adult chicken, cultivated for 72 hours in equal volumes of plasma and embryonic juice. Camera lucida drawing made 24 hours after first passage into fresh medium.

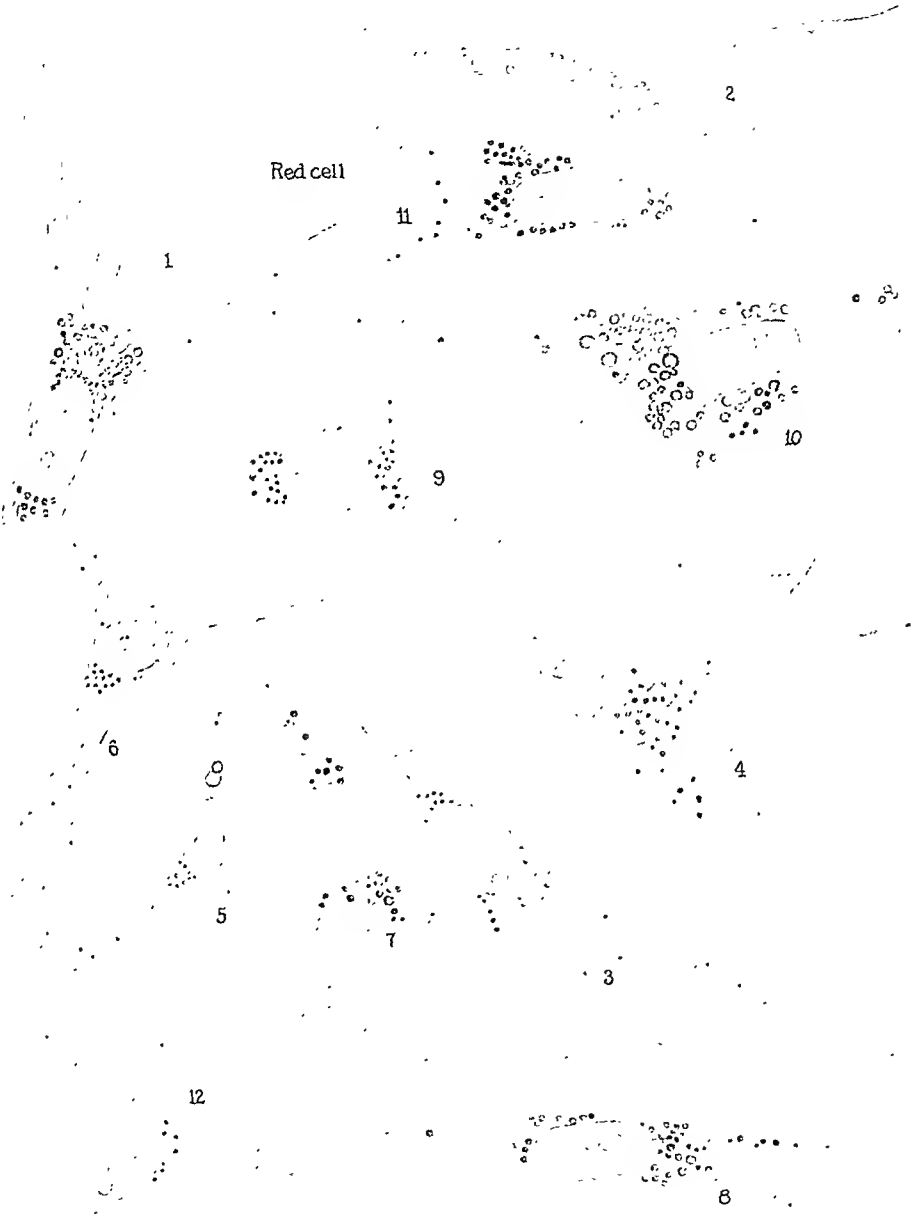
FIG. 8. Fibroblasts from subcutaneous connective tissue of an adult chicken, cultivated for 5 days in equal volumes of plasma and embryonic juice. Camera lucida drawing made 24 hours after second passage into fresh medium.

FIG. 9. Fibroblasts from subcutaneous connective tissue of a tumor chicken, cultivated for 72 hours in equal volumes of plasma and embryonic juice. Camera lucida drawing made after 24 hours incubation.

FIG. 10. Fibroblasts from subcutaneous connective tissue of a tumor chicken, cultivated for 13 days in equal volumes of plasma and embryonic juice. Camera lucida drawing made 24 hours after third passage into fresh medium.

FIG. 11. Fibroblasts from same strain of connective tissue as shown in Fig. 10, cultivated for 17 days in equal volumes of plasma and embryonic juice. Camera lucida drawing made after 48 hours incubation.

FIG. 12. Fibroblasts from subcutaneous connective tissue of an adult chicken, cultivated for 6 days in plasma containing a trace of embryonic juice. Camera lucida drawing made after 48 hours incubation.



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Edited by

W. J. CROZIER

JOHN H. NORTHROP

W. J. V. OSTERHOUT

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order to determine whether metabolic changes can modify their cytoplasmic structures.

In a first series of experiments, fragments of leucocytic films and flaps of subcutaneous connective tissue from adult chickens were cultivated in a medium composed of plasma, Tyrode solution, and embryonic juice. After a period varying from 2 hours to a number of days, fragments of the colonies were removed from the flasks, transferred to cover-glasses, and stained with neutral red and Janus green. Camera lucida drawings were made at a magnification of 1600 diameters.

Experiment 1. Monocytes Freshly Removed from the Blood.—Culture 3002-A2, Jan. 14, 1926. Fragments of leucocytic films placed on cover-glasses in a hanging drop of plasma and embryo juice. After 2 hours, preparation stained; camera lucida drawings. A few monocytes are seen among many polymorphonuclear leucocytes. Length of cells, 10 to 15 μ ; active pseudopods; approximately 10 small neutral red vesicles about 1 μ in diameter, containing 1 or 2 small granules; vesicles grouped around the centriole or scattered through the cytoplasm.

Experiment 2. Blood Monocytes after 2, 24, 48, 72, and 96 Hours Cultivation.—Culture 8740-D, Apr. 19. Fragments of leucocytic film cultivated on cover-glasses in equal parts of plasma and embryo juice. After 2 hours, preparation stained; camera lucida drawings. Large number of polymorphonuclear leucocytes (Fig. 1) migrating into the medium among a few red blood corpuscles (Fig. 2); no lymphocytes; some monocytes, all of identical appearance; cells 10 to 15 μ long; 8 or 10 neutral red vesicles about 1 μ in diameter, radially arranged around the centriole in a few cases; mitochondria as faintly stained dots; no fat globules (Fig. 3). Apr. 20, stained; camera lucida drawings. Active polymorphonuclear leucocytes, unchanged in size or appearance; a few lymphocytes. Without exception, all the monocytes have increased in size, being 30 to 40 μ in length; no change in the mode of locomotion and in the general appearance of the cells. The neutral red vesicles are more numerous, from 0.25 to 1.5 μ in diameter, and contain granules; no rosette disposition; mitochondria more deeply stained, assuming the shape of rods (Fig. 5). Apr. 21, stained; camera lucida drawings. Active polymorphonuclear leucocytes, unchanged in size or appearance; a few lymphocytes. All the monocytes have increased in size, being 40 to 45 μ in length; no change in the mode of locomotion and in the general appearance of the cells; no rosette disposition of the vesicles. They are numerous, 0.5 to 2 μ in diameter, and deeply stained. A number of the cells show partially digested polymorphonuclear leucocytes; mitochondria as round and rod-shaped granules, deeply stained (Fig. 6). Apr. 22, stained; camera lucida drawings. Decrease in the number of polymorphonuclear leucocytes. They are unchanged in size or appearance; no lymphocytes. Many of the monocytes are slender, 50 to 75 μ in length; no change in the mode of locomotion.

THE FUNDAMENTAL PROPERTIES OF THE FIBROBLAST AND THE MACROPHAGE.

II. THE MACROPHAGE.

BY ALEXIS CARREL, M.D., AND ALBERT H. EBELING, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

PLATES 8 AND 9.

(Received for publication, May 12, 1926.)

The purpose of the experiments described in this article was to elucidate the relations of the macrophage¹ and the monocyte,² and to define these cells by their essential biological properties.

Monocyte and Macrophage as Functional Variations of a Single Type.

The macrophages which migrated from fragments of connective tissue, organs, tumors, etc., of adult animals did not differ markedly from the blood monocytes which had been cultivated *in vitro* for a few days. This fact rendered it probable that both cells merely represent metabolic states of a single type, and that their structure can be modified at will by certain changes in the composition of the medium. Two convergent methods were used. The first one consisted of placing macrophages derived from the subcutaneous connective tissue and monocytes obtained from the blood in media of identical composition, and ascertaining the structural changes occurring after a few days. If monocytes and macrophages merely express different metabolic states of a single cell, they must become identical in appearance when living under the same conditions. By the second method, monocytes and macrophages were studied in media of different composition in

¹ Synonyms: round rhagiocrin cells of Renaut; polyblasts of Maximow; clasmatoocytes of Ranvier; endothelial leucocytes of Mallory; adventitial cells of Marchand; pyrrol cells of Goldmann.

² Synonyms: large mononuclear leucocytes of Ehrlich; endothelial leucocytes of Mallory; blood histiocytes of Aschoff.

Experiment 7. Tissue Macrophages after 24 Hours Cultivation.—Culture 2956-C5, Jan. 14. Fragments of subcutaneous connective tissue from an adult chicken cultivated on cover-glasses in equal parts of plasma and embryo juice. Jan. 15, stained; camera lucida drawings. Long, slender macrophages; very active flagellate pseudopods; length of cells, 60 to 85 μ ; about 8 neutral red vesicles, 0.5 to 2 μ in diameter, containing 1 or 2 small neutral red granules.

Experiment 8. Tissue Macrophages after 24 Hours Cultivation.—Culture 3097-C, Jan. 28. Fragments of subcutaneous connective tissue from an adult chicken cultivated on cover-glasses in equal parts of plasma and embryo juice. Jan. 29, stained; camera lucida drawings. Diameter of cells, 30 μ ; approximately 30 moderately stained neutral red vesicles, 0.5 to 3 μ in diameter. Most of the vesicles contain coarse, dark red granules—which move more or less actively; many small fat globules between the vesicles. After a few minutes, the fluid in the vacuoles takes up the stain gradually and becomes orange, yellow, or red; vesicles not arranged around the centriole, but distributed through the trophoplasm; active folding and unfolding of the kinoplasm.

Experiment 9. Tissue Macrophages after 24 Hours Cultivation.—Culture 3777-C, Apr. 22. A fragment of subcutaneous connective tissue from a normal adult chicken cultivated on cover-glasses in equal parts of plasma and embryo juice. Apr. 23, migration of a large number of macrophages; stained; camera lucida drawings. Diameter of cells, 40 μ ; active kinoplasm. Trophoplasm contains about 20 neutral red vesicles, 0.25 to 2 μ in diameter, showing rosette disposition; small fat globules around nucleus and vesicles; mitochondria deeply stained, globular, and rod-shaped (Fig. 9).

Experiment 10. Tissue Macrophages after 24 Hours Cultivation.—Culture 3790-C, Apr. 23. A fragment of subcutaneous connective tissue from a normal adult chicken cultivated in equal parts of plasma and embryo juice. Apr. 24, migration of moderate number of macrophages; stained; camera lucida drawings. Length of cells, 75 μ ; active kinoplasm; trophoplasm filled with neutral red vesicles, 0.25 to 2 μ in diameter; no fat globules; mitochondria deeply stained, globular, and rod-shaped (Fig. 10).

In a second series of experiments, blood monocytes and fragments of subcutaneous connective tissue from adult chickens were cultivated in a control medium composed chiefly of serum, and an experimental medium of Tyrode or Ringer solution. The cover-glass preparations were stained with neutral red or neutral red and Janus green.

Experiment 1. Effect of Tyrode Solution and Serum on Monocytes.—Culture 3386-C, Mar. 2. Fragments of leucocytic film cultivated in flasks in the ordinary medium composed of 0.5 cc. plasma, 0.5 cc. embryo juice, and 1 cc. Tyrode solution. Mar. 3, cultures washed with 2 cc. of Tyrode solution, and patched with 25 cc. plasma and 25 cc. embryo juice. Mar. 4, cultures washed in 2 cc. Tyrode

tion. The vesicles are very numerous, 0.5 to 2 μ in diameter, and fill the trophoplasm. In some cases, cells show ingested red blood corpuscles and polymorphonuclear leucocytes; mitochondria as deeply stained granules (Fig. 7). Apr. 23, stained; camera lucida drawings. Very few active polymorphonuclear leucocytes. They are unchanged in size or appearance; no lymphocytes. Most of the monocytes are slender, 55 to 80 μ in length. The undulating membrane has disappeared along the sides of the cells. The vesicles are 0.25 to 1 μ in diameter, less numerous, and not as deeply stained as in the cells observed after 3 days incubation; no evidence of ingested cells. Mitochondria as deeply stained granules (Fig. 8).

Experiment 3. Blood Monocytes after 2 and 24 Hours Cultivation.—Culture 8741-D, Apr. 20. Fragments of leucocytic film cultivated on cover-glasses in equal parts of plasma and embryo juice. After 2 hours, preparation stained; camera lucida drawings. Normal polymorphonuclear leucocytes; all monocytes identical in appearance; cells 10 to 15 μ long; 8 or 10 neutral red vesicles, showing the rosette arrangement in some cases; mitochondria as faintly stained dots; no fat globules; cells identical with those of Fig. 4. Apr. 21, stained; camera lucida drawings. Active polymorphonuclear leucocytes; a few lymphocytes. Every visible monocyte has increased in size, being 30 to 40 μ in length; same mode of locomotion and general appearance; rod-shaped mitochondria. The neutral red vesicles are larger and contain dark granules; not grouped around the centriole; cells identical with those of Fig. 5.

Experiment 4. Blood Monocytes Cultivated for 7 Days.—Culture 3020-A2, Jan. 21. Fragments of leucocytic film cultivated in a flask containing 0.5 cc. plasma, 1 cc. Tyrode solution, and 0.5 cc. embryo juice. After coagulation, 0.5 cc. serum and 0.5 cc. embryo juice added to medium. Jan. 22, washed in Tyrode solution, and fresh medium added. Jan. 25, same treatment. Jan. 28, fragments of coagulum transferred to cover-glasses and stained; camera lucida drawings. Length of cells, 30 μ ; active kinoplasm; trophoplasm filled with large number of neutral red vesicles, deeply stained, resembling berries; diameter of vesicles, 1 to 4 μ . Vesicles contained many small granules, irregular in shape, and darkly stained; no rosette arrangement.

Experiment 5. Blood Monocytes Cultivated for 13 Days.—Culture 2954-C, Jan. 7. Fragments of leucocytic film from normal chickens cultivated on cover-glasses in equal parts of plasma and embryo juice; three passages into fresh medium. Jan. 19, stained; camera lucida drawings. Very long and slender monocytes; active kinoplasm; length of cells, 60 μ ; 15 neutral red vesicles, 0.5 to 2 μ in diameter. Some of the vesicles contain 1 to 5 small red granules.

Experiment 6. Tissue Macrophages after 24 Hours Cultivation.—Culture 3136-C5, Feb. 1. A fragment of subcutaneous connective tissue from a normal adult chicken cultivated on cover-glasses in equal parts of plasma and embryo juice; migration of a few cells. Feb. 2, stained; camera lucida drawings. Length of cells, 30 μ ; active kinoplasm. Trophoplasm contains 8 or 10 neutral red vesicles, 1 to 2 μ in diameter, each vesicle having several large red granules, generally agglutinated together; some fat globules between the vesicles.

1. Monocytes in Ringer solution. Elongated and very active cells, 25μ in length; about 15 faintly stained neutral red vesicles, 0.25 to 1.25μ in diameter, scattered through the trophoplasm, and with no granules; mitochondria as small dots, faintly stained (Fig. 15).

2. Monocytes in serum. Very active cells, with several branches, 40μ in length; deeply stained neutral red vesicles, 0.25 to 1.25μ in diameter, without granules, arranged around centriole; globular and rod-like mitochondria, well stained (Fig. 16).

Experiment 5. Effect of Tyrode Solution and of Blood Serum on Monocytes.—Culture 8496-D, Feb. 1. Fragments of culture of monocytes 3030-A2 are placed in flasks, containing 1.5 cc. plasma and 0.5 cc. embryo juice. After coagulation, Flask 1 washed for 10 minutes in 4.5 cc. of Tyrode solution. After the fluid is removed, 1.5 cc. of Tyrode solution is introduced into the flask. Flask 2 is left undisturbed. Feb. 2, Flask 1 washed with Tyrode solution for 10 minutes. Feb. 3, Flask 1 washed for 5 minutes with Tyrode solution; surface of coagulum of Flask 2 washed for a few seconds in Tyrode solution, and 0.5 cc. serum introduced. In Flask 1, monocytes are elongated, transparent, and in comparatively small numbers. In Flask 2, they are shorter, less transparent, very numerous, and invading the medium actively. Feb. 5, fragments from Flask 2 transferred to cover-glasses; stained; camera lucida drawings. Feb. 6, fragments from Flask 1 transferred to cover-glasses; stained; camera lucida drawings.

1. Monocytes in Tyrode solution. Very active cells, 35μ in length; small segregation apparatus; a few neutral red vesicles, 0.25 to 0.75μ in diameter, faintly stained; no granules; few fat globules.

2. Monocytes in serum. Very active cells, 50μ in length; very large segregation apparatus; many neutral red vesicles, 0.5 to 2μ in diameter, resembling a mass of darkly stained berries; no fat globules.

Experiment 6. Effect of Tyrode Solution and Blood Serum on Monocytes.—Culture 3511-C, Mar. 16. Fragments of leucocytic film cultivated in the ordinary manner. Mar. 18, cultures washed with 2 cc. Tyrode solution for 10 minutes, 1 cc. Tyrode solution introduced into Flask 1, and 1 cc. of 50 per cent serum into Flask 2. Mar. 19, cultures washed with 2 cc. Tyrode solution for 10 minutes, and fresh fluid introduced into each flask. Mar. 20, fragments transferred to cover-glasses; stained; camera lucida drawings.

1. Monocytes in Tyrode solution. Cells, 25μ in diameter; active kinoplasm; faintly stained neutral red vesicles, 0.25 to 0.5μ in diameter; few mitochondria, faintly stained; a few small fat globules.

2. Monocytes in serum. Length of cells, 55μ ; neutral red vesicles, 0.25 to 3μ in diameter, more deeply stained; mitochondria well stained; a number of small fat globules scattered about among the vesicles.

Experiment 7. Effect of Tyrode Solution and Serum on Monocytes.—Culture 3020-A, Jan. 21. Fragments of leucocytic film cultivated in the ordinary manner. After coagulation, 1 cc. Tyrode solution introduced into Flask 1, and 0.5 cc. serum and 0.5 cc. embryo juice into Flask 2. Jan. 22, cultures washed with 2 cc. Tyrode

solution, which is removed after 10 minutes; Flask 1, 1 cc. Tyrode solution introduced, and Flask 2, 0.5 cc. serum and 0.5 cc. Tyrode solution. Mar. 6, cultures washed in Tyrode solution and fresh fluid introduced into the flasks. Mar. 8, fragments of coagulum transferred to cover-glasses; stained; camera lucida drawings.

1. Monocytes in Tyrode solution. Length of cells, 32μ ; active kinoplasm; trophoplasm filled with faintly stained neutral red vesicles containing no granules; between the vesicles, some fat globules and faintly stained mitochondria. The latter are small dots, hardly visible (Fig. 11).

2. Monocytes in serum. Very active multibranching cells, 50μ in length; kinoplasm packed with smaller neutral red vesicles, deeply stained, resembling berries; practically no fat globules; well stained mitochondria, globular, rod-shaped, and filamentous (Fig. 12).

Experiment 2. Effect of Tyrode Solution on Monocytes.—Culture 3079-A1, Feb. 18. Monocytes which have grown in the ordinary medium since Feb. 16 are washed for 10 minutes with 2 cc. Tyrode solution. After its removal, 1 cc. of Tyrode solution is introduced into the flask. Feb. 21, same treatment. Feb. 24, fragment of coagulum transferred to cover-glasses; stained; camera lucida drawings. Macrophages are of small size with very active kinoplasm; length of cells, 25μ . The area occupied by the neutral red vesicles and the nucleus is very small. About 15 small neutral red vesicles, 0.25 to 1μ , are grouped around the centriole. Between them and the centriole is an area lightly stained in red and composed of small granules; rosette arrangement; faintly stained mitochondria as small dots around the nucleus and the neutral red vesicle; no fat.

Experiment 3. Effect of Ringer Solution and Serum on Monocytes.—Culture 3445-C, Mar. 9. Preparation of the cultures in the ordinary medium. Mar. 11, cultures washed in 2 cc. Ringer solution. After the removal of the fluid, 1 cc. Ringer solution is introduced into Flask 1, and 0.5 cc. serum and 0.5 cc. Ringer solution into Flask 2. Mar. 12 and 13, same treatment. Mar. 15, fragments of coagulum transferred to cover-glasses; stained; camera lucida drawings.

1. Monocytes in Ringer solution. Small cells, 25μ in length; 25 pale vesicles, 0.25 to 1μ in diameter, and faintly stained mitochondria; large number of small fat globules; active kinoplasm (Fig. 13).

2. Monocytes in serum. Cells active and larger, 50μ in length; area occupied by the neutral red vesicles, 0.25 to 3μ in diameter, containing a fluid more deeply stained than in cells cultivated in Ringer solution; few fat globules; large number of mitochondria (Fig. 14).

Experiment 4. Effect of Ringer Solution and Serum on Monocytes.—Culture 3471-C, Mar. 11. Preparation of the cultures by the ordinary method. Mar. 13, cultures washed in 2 cc. Tyrode solution. After removal of the fluid, 1 cc. of Ringer solution is introduced into Flask 1, and 0.5 cc. serum and 0.5 cc. Ringer solution into Flask 2. Mar. 15, cultures washed and fresh medium introduced. Mar. 16, fragments of coagulum transferred to cover-glasses; stained; camera lucida drawings.

reaching 30 to 40 μ . The neutral red vesicles were augmented in number and size, and filled with orange-red fluid containing small or large dark granules. No fat globules were seen. The mitochondria assumed the appearance of rods or short filaments. Every monocyte present in the preparation was transformed in this manner. The change became more marked after 48 hours (Fig. 6). The trophoplasm was filled with debris of polymorphonuclear leucocytes. After 72 hours (Fig. 7), the size of the cells reached 75 μ . The segregation apparatus and the mitochondria showed a corresponding increase. The undulating membrane was very active. The polymorphonuclear leucocytes were normal (Fig. 1). However, a number of them and also some red blood corpuscles had been phagocyted and were being digested by the monocytes. All the monocytes present in the preparation had become large cells, indistinguishable from tissue macrophages. After 96 hours cultivation (Fig. 8) the polymorphonuclear leucocytes, lymphocytes, and red corpuscles had practically disappeared from the preparation, and the segregation apparatus of the monocytes had slightly decreased in size. The undulating membrane moved only at the ends of the cells.

The tissue macrophages were always larger than the monocytes just obtained from the blood. Their size varied from 30 to 85 μ . The undulating membrane and its flagellate-like folds moved in the same manner as those of the monocytes. The number of the neutral red vesicles varied from 15 to 30 or more. They were often grouped around the centriole in a typical rosette. Their diameter varied from 1 to 2 μ . Generally, they contained 1 or 2 small red granules. A few fat globules were seen between the vesicles. The mitochondria were located around the nucleus and the mass of the vesicles, and assumed the shape of dots and rods (Figs. 9 and 10).

As these results clearly show, blood monocytes increase in size when cultivated in plasma and embryo juice in the presence of polymorphonuclear leucocytes, and in 24 hours they reach the dimensions of the tissue macrophages. At the same time that the size and proliferating activity of the cells are augmented, the segregation apparatus becomes larger, dark stained granules appear in the vesicles, and the mitochondria assume the shape of rods and short filaments. The rosette arrangement of the neutral red vesicles around the centriole

solution for 10 minutes, and after removal of the fluid, patched with 1 cc. of 0.5 cc. plasma and 0.5 cc. embryo juice. After coagulation, fresh fluid added to each flask. Jan. 23, same treatment. Jan. 27, fragments from Flask 1 transferred to cover-glasses; stained; camera lucida drawings; Flask 2, ordinary treatment. Jan. 28, fragments from Flask 2 transferred to cover-glasses; stained; camera lucida drawings.

1. Monocytes in Tyrode solution. Length of cells, 30μ ; small vesicular apparatus; about 20 neutral red vesicles, 0.25 to 1.5μ in diameter, faintly stained; no granules in the vesicles; about 15 fat globules.

2. Monocytes in serum and embryo juice. Length of cells, 40μ ; about 40 neutral red vesicles, 0.25 to 2.5μ in diameter, many of them containing red granules.

Experiment 8. Effect of Tyrode Solution on Tissue Macrophages.—Culture 3841-C, Apr. 29. Fragments of subcutaneous connective tissue from an adult chicken cultivated on cover-glasses in equal parts of plasma and embryo juice. Apr. 30, extensive migration of macrophages into medium; one preparation stained; camera lucida drawings. Diameter of cells, 45μ ; many neutral red vesicles, 0.25 to 3.5μ in diameter; rosette disposition; no fat globules; numerous mitochondria, well stained granules and short rods grouped around nucleus and vesicular area (Fig. 17).

Other preparations transferred to a flask containing 0.5 cc. plasma, 1 cc. Tyrode solution, and 0.5 cc. embryo juice. After coagulation, culture washed in 2 cc. Tyrode solution for 10 minutes; fluid removed, and 1 cc. Tyrode solution introduced into the flask. May 1, a few active cells migrating into the fresh medium; washed in 2 cc. Tyrode solution, and fresh Tyrode solution introduced. May 3, same treatment. May 5, fragments of coagulum transferred to cover-glasses; stained; camera lucida drawings. Very active cells, 25μ in diameter; neutral red vesicles, 0.25 to 1.5μ in diameter; rosette disposition; no fat globules; mitochondria well stained granules with a few rods grouped around nucleus and vesicular area (Fig. 18).

The results of these two series of experiments may be summarized as follows:

In the first series, blood monocytes and tissue macrophages were cultivated in identical media for various periods of time. The monocytes, examined 2 hours after the preparation of the cultures (Figs. 3 and 4), were 10 to 15μ in length. Their undulating membrane moved unceasingly and showed its folds as flagellate pseudopods. The neutral red vesicles, 6 to 10 in number, were radially arranged around the centriole, or scattered irregularly through the trophoplasm. The mitochondria were small and round (Figs. 3 and 4). After 24 hours (Fig. 5), the size of the cells increased, their length

organs. Cell morphology is determined by, and varies according to, the chemical composition of the pericellular fluid.

Mode of Locomotion of the Macrophage and Structure of Its Kinoplasm.

The mode of locomotion of the macrophage was studied on cinematographic films of cultures of blood, connective tissue, and Rous sarcoma. Both direct light and dark-field illumination were used. The photographs were taken at a speed of 60 exposures per minute and at a magnification of 400 diameters.³ As the films were projected at a speed of 10 or 11 exposures per second, the rate of motion of the cells was increased approximately ten times. It was thus possible to observe simultaneously on the films the progression of the cells and the movements of their organs. The small monocytes observed in freshly prepared cultures moved more slowly than the polymorphonuclear leucocytes, and more rapidly than the lymphocytes. The mode of progression of the three types of cells differed widely. The lymphocytes sent forth short, blunt pseudopods, and advanced like small worms. The polymorphonuclear leucocytes projected forward long arms, into which the rods streamed. Often they dragged a thin tail behind them. While the polymorphonuclear leucocytes moved like amebæ, the monocytes assumed an appearance more nearly comparable to that of an octopus. Around their body and processes, thread-like pseudopods moved unceasingly. They could be distinguished at first sight from the polymorphonuclear leucocytes and lymphocytes by their peculiar way of carrying themselves. After the monocytes had assumed the appearance of macrophages, their mode of locomotion was not modified. The movements of both monocytes and macrophages growing from adult connective tissue were identical, and neither cell could be distinguished from the other.

The study of macrophages photographed by direct light and by dark-field illumination led to a clear conception of their structure. These cells are composed, as is well known, of a central body or trophoplasm, and a marginal portion or kinoplasm. The trophoplasm contains the nucleus, the mitochondria, and the neutral red vesicles. Although the cell body is plastic, and the nucleus as well as the cyto-

³ The cultures were filmed by Mr. Heinz Rosenberger.

is present in the tissue macrophages, as well as in the monocytes. However, it is not a constant characteristic of either of these cells. There is no difference between the mitochondria of monocytes and of macrophages, after the monocytes have been cultivated in plasma and embryo juice. As all the large mononuclear cells present in the medium exhibit a similar appearance and size, they certainly derive from the monocytes observed in the fresh cultures. It is obvious that, when cultivated under the same conditions, the blood monocytes become indistinguishable from the macrophages of adult connective tissue (Figs. 7 to 10).

In the second series of experiments, macrophages originating from monocytes and tissue macrophages were placed in nutrient and non-nutrient media, in order to ascertain whether their morphology could be modified by a nutritional change. It is known that serum is a nutrient medium for the monocytes, whereas they starve in Tyrode or Ringer solution. The control monocytes, which were kept in serum, continued to resemble tissue macrophages (Figs. 12, 14, and 16). The segregation apparatus was well developed and the vesicles often looked like red berries and contained some granules. The mitochondria appeared as short filaments, deeply stained with Janus green. The cells remained large and very active. Their length varied from 40 to 70 μ . The experimental monocytes were deprived of food in flasks containing only Tyrode or Ringer solution as a fluid medium (Figs. 11, 13, and 15). Their size decreased, and the nucleus also became smaller. At the same time, the segregation apparatus lost its importance, the granules disappeared from the vesicles which were filled with pale fluid, and the mitochondria became shorter, less numerous, and faintly stained. Some fat globules appeared between the vesicles. It was evident that in the cells subjected to starvation, the neutral red vesicles and the mitochondria became profoundly modified. Similar phenomena were observed in tissue macrophages cultivated in a medium composed chiefly of Tyrode solution (Figs. 17 and 18). They reverted to a smaller type, markedly resembling monocytes.

Monocytes and tissue macrophages do not differ essentially from one another. They represent functional states of a single type, which readily responds to a change in nutrition by modifying its protoplasmic

extract, ameboid cells migrate in large numbers into the coagulum. The polymorphonuclear leucocytes, which move rapidly, occupy the outer zone of the invaded area, while the inner zone consists chiefly of the slower monocytes which later multiply and scatter everywhere. Eventually, small islands composed of monocytes appear at a distance from the main colonies. The monocytes rapidly phagocyte the polymorphonuclear leucocytes and in 3 or 4 weeks spread on the entire medium in a more or less regular formation. They do not build up a tissue, as fibroblasts do. Sometimes they unite as chains. They never come into contact at their sides. Instead of remaining packed together, they disperse through the whole coagulum. They may congregate around some foreign bodies, such as fragments of muscle or protein precipitate, which they devour. But as soon as the food is exhausted, they scatter again. When the original fragments of the leucocytic film have been removed, the coagulum appears as homogeneous. As long as the cells remain normal, the flask seems to be empty. When the segregation apparatus develops abnormally, the colonies may look like whitish spots on the transparent background. If death occurs, the colonies become opaque. Digestion of the coagulum does not take place even after the death of the cells. As a rule, digestion of the coagulum down to the glass is a characteristic of the malignant transformation of the cells after inoculation with Rous virus. The clot seems to be moth-eaten, and on the edges of the digested areas small masses of necrotic tissue may be seen. Simultaneously, some cells assume the appearance of fibroblasts. The transformation of the macrophages into fibroblasts never occurs spontaneously when the cells are allowed to migrate freely, as they do in a normal culture and when no sarcoma virus is present. If macrophages crowd together through the action of some mechanical factor, or following inoculation with Rous virus, they die or become transformed into fibroblasts.

Colonies of tissue macrophages were obtained from the subcutaneous connective tissue of adult chickens, and from other normal or pathological tissues. Their appearance and behavior have always proved to be identical with those of the monocytes.

plasmic organs glide nimbly into the advancing process during the progression of the cell, it is relatively immobile when compared to the kinoplasm. The kinoplasm is the peripheral and thin cytoplasm from which thread-like pseudopods seem to extend themselves. These filiform appendages move with great activity. When the flagellated pseudopods are examined on films taken with dark-field illumination, they appear to be the folds of an extremely thin membrane which projects itself from the kinoplasm and is a part of it. This membrane undulates incessantly, like a delicate silk veil when blown by the wind. Under direct light, the membrane becomes invisible. Its folding and outer edges only are then seen, to be mistaken for thread-like pseudopods. When the macrophage stops progressing and assumes a circular shape, it appears surrounded by the membrane which completely unfolds itself and moves like the waves of the sea on a sandy shore. Under dark-field illumination, the presence of the membrane is detected because its surface as well as its folds become lighted. When, in the course of a slow undulation, the convex surface of a fold is illuminated from the side, the membrane is seen as an extremely thin structure covering part or all of the periphery of the cell, and molding itself upon any impinging bodies, fibrin, bacteria, and other cells. When a lymphocyte approaches a macrophage, it is rapidly enveloped by the foldings and appears to glide into them toward the body of the cell, where it is digested. The incessant motion of the undulating membrane is probably the cause of the inability of the macrophages to unite and form a tissue as fibroblasts do. Possibly it is an essential function of the cytoplasm, and its suppression, when cells are crowded, may bring about death.

Macrophages obtained from the subcutaneous connective tissue of adult chickens were studied by the same method, and found to move exactly as do monocytes. Normal blood monocytes, large monocytes growing in flasks, sarcoma cells, and tissue macrophages possess an undulating membrane. It is a characteristic common to the four groups of cells.

Characteristics of the Colonies of Macrophages.

When fragments of a leucocytic film are placed in a medium composed of 0.5 cc. plasma, 1 cc. Tyrode solution, and 0.5 cc. embryonic

protein. Fragments of muscle, killed by heating or freezing and placed in the immediate vicinity of a colony of monocytes, are soon invaded by migrating cells which creep along them and begin their work of erosion. The muscle fibers assume the appearance of worm-eaten logs. They finally disintegrate under the repeated attacks of the monocytes, which migrate through the medium heavily charged with debris. As long as the cells congregate around the muscle fibers, they remain very large. After the supply of food is exhausted, they become smaller. A similar phenomenon takes place when drops of a protein precipitate are deposited near a group of monocytes. It seems as if discrete particles of proteins are better food than proteins in solution. However, when the fluid medium contains a minute amount of proteose, the rate of proliferation is also markedly augmented. If a larger concentration of proteose is used, the monocytes accumulate many fat globules and neutral red vacuoles, and die after a short time. It is significant that a concentration of proteose, which greatly increases the rate of growth of the fibroblasts, brings about the death of the macrophages.

Monocytes are endowed with a peculiar resistance to the action of arsenous oxide. When they are cultivated for 24 hours in a medium containing arsenous oxide at a concentration of $1/180,000$, they die in large numbers. However, some of the cells resist the poison and multiply actively. A concentration of $1/200,000$ is much less injurious. The cells proliferate in spite of having been treated by arsenous oxide, and after a few days the cultures assume a normal appearance. While macrophages resist arsenous oxide at a concentration of $1/200,000$, fibroblasts are killed when their medium contains arsenous oxide at a concentration of $1/800,000$. If fragments of Rous sarcoma are placed in a medium containing $1/400,000$ arsenous oxide, the fibroblasts and the degenerating sarcoma cells rapidly disappear and only normal macrophages survive. Tissue macrophages possess approximately the same food requirements and the same susceptibility to arsenous oxide as blood monocytes.

DISCUSSION.

The essential properties of the blood monocyte and the tissue macrophage appear to be identical, as shown by the appearance of the

Rate of Growth of the Colonies.

In a medium composed chiefly of blood serum, the rate of multiplication and migration is such that monocytes originating from fragments of a film about 3 by 2 mm. entirely cover the coagulum of a flask 5 cm. in diameter in approximately 3 weeks. The rate of growth of the colony may be appraised in terms of the time the cells take to invade the culture medium and the number of cells contained in the unit of surface. However, no method has yet been developed by which the rate of multiplication may be accurately measured. Only considerable differences can be appreciated. There is no difficulty in detecting the variations in the rate of multiplication of macrophages or monocytes in media widely different in nature. When colonies of monocytes are cultivated in a hanging drop and transferred to a new medium every 2 or 3 days, the size of the colonies generally doubles in 4 or 5 days. The velocity of the multiplication of the cells is probably less great than that of epithelial cells and fibroblasts.

Food Requirements of Macrophages. Their Susceptibility to Arsenous Oxide.

Monocytes and macrophages do not feed on egg albumin, nucleic acid, amino acids, crystallized egg albumin, egg yolk, or pure embryo juice. They have the property of living and multiplying in blood serum and differ from fibroblasts and epithelial cells, which die in blood serum and multiply indefinitely in embryo juice. Although they may proliferate for several weeks when cultivated in plasma or serum, it is far from certain that they reach their optimum metabolic activity in this medium. In fresh blood cultures, monocytes almost immediately phagocyte polymorphonuclear leucocytes and red corpuscles, and their length increases from 10 or 15 μ to 70 or 80 μ . When the food is exhausted, they generally revert to a smaller type, even in the presence of blood serum. The increase in size of the cells during the first days of cultivation is probably due to the abundant food supplied by polymorphonuclear leucocytes, lymphocytes, and red corpuscles. Monocytes and macrophages also absorb with avidity minute fragments of dead muscle, or particles of precipitated

non-nutrient media. The starved tissue macrophages grew smaller (Fig. 18), lost part of their segregation apparatus, reduced their mitochondria, and became almost similar to monocytes freshly removed from the blood. It is obvious that macrophages can revert to a form closely resembling monocytes, when they are deprived of food. These facts completed the demonstration that monocytes and macrophages are the expression of various metabolic states of a single type. Monocytes, macrophages, and fibroblasts are closely related forms. While the transformation of a monocyte into a macrophage is easily brought about by an abundant food supply, that of a macrophage into a fibroblast requires the presence of substances of unknown nature, probably set free by dead cells.⁵

Between monocytes and macrophages, the main difference is one of size (Figs. 3 and 10). In both cells, the mode of locomotion and the movement of the cytoplasmic organs are identical. Both cells are surrounded by an undulating membrane which moves unceasingly (Figs. 6 and 9). The external boundary of this appendage is irregular and invisible with direct light. This characteristic distinguishes the macrophage from the fibroblast which is limited by a sharp line of geometric design. The surface of the fibroblast is rigid, and the motion of the protoplasm takes place only at the end of the processes. When a macrophage begins to lose its activity, its sides become limited by a sharp, regular line, while the undulating membrane remains present at both ends (Fig. 8). The structural characteristics of fibroblasts and macrophages which are clearly noted in cinematographic films explain the differences observed in the constitution of the colonies. The fibroblasts that move only through their end processes can come into close contact at their sides. They form circular colonies where each component cell flows radially toward the periphery. On the contrary, macrophages are incapable of remaining packed together because they are surrounded by a membrane which moves continuously. They progress far more rapidly than the fibroblasts, and scatter through the coagulum without forming any dense colonies.

Both macrophages and fibroblasts possess a segregation apparatus which increases in size when the cells receive an abundant food supply

⁵ Carrel, A., and Ebeling, A. H., *J. Exp. Med.*, 1926, xliii, 461.

colonies, their action on the medium, the mode of locomotion and the structure of the cells, their rate of growth, their food requirements, and their susceptibility to certain toxic substances. The cells differ slightly in the size and development of their segregation apparatus and mitochondria (Figs. 3 to 10). The radial arrangement of the neutral red vacuoles around the centriole, or rosette disposition, considered by Sabin, Doan, and Cunningham⁴ as being characteristic of the monocyte (Figs. 3 and 16), is frequently observed also in the tissue macrophage (Figs. 9, 17, and 18). In normal macrophages, as well as in monocytes, mitochondria are always present (Figs. 12 and 17). Monocytes and macrophages cultivated in media of identical composition become identical in structure. There is no difference between a monocyte that has been kept for a few days in plasma and embryo juice, and a tissue macrophage (Figs. 5, 7, and 10). Both cells have approximately the same size; their segregation apparatus is well developed; the neutral red vesicles may be arranged in a rosette around the centriole or scattered through the cytoplasm; and the mitochondria appear as short filaments. The disposition of the neutral red vesicles around the centriole, their size and number, the condition of the mitochondria, and the presence of the fat globules, are determined by the composition of the culture medium and not by the origin of the cells. A monocyte supplied with an abundance of food in a solid medium becomes a macrophage (Fig. 7). A macrophage is merely a monocyte in a more active metabolic condition.

The polymorphism of the cell was further ascertained by experiments in which macrophages derived from monocytes or obtained from adult connective tissue were caused to revert to a less active metabolic state. When blood macrophages (Figs. 12, 14, and 16) were cultivated in Tyrode or Ringer solution, the starved cells became smaller (Figs. 11, 13, and 15), the rod-like mitochondria assumed the appearance of dots, the neutral red vesicles decreased in number and diameter, and their segregation granules disappeared. A similar phenomenon was observed when tissue macrophages (Fig. 17) from the subcutaneous connective tissue of adult chickens were cultivated in nutrient and

⁴ Sabin, F. R., Doan, C. A., and Cunningham, R. S., *Carnegie Institution of Washington, Pub. No. 361, Contributions to Embryology*, 1925, xvi, 125.

found in the subcutaneous connective tissue. In the blood, as well as in the tissues, the structure of the mononuclear cell depends upon the composition of its medium. It is probable that the nutrition of spherical monocytes in suspension in a fluid is not as active as when the cells, stretching themselves on the fibrin framework of a coagulum, greatly increase their surface. The only response that the large mononuclear can make to the physicochemical conditions of the circulating blood is probably to assume a monocytic form. On the other hand, when monocytes migrate from the capillaries into the tissues, they automatically become transformed into macrophages, as they do in a solid medium containing an abundant food supply.

The abnormal multiplication of monocytes and macrophages in certain diseases is probably due to an increase in their food supply. The formation of an exudate richer in proteins than normal lymph, the death of leucocytes, lymphocytes, or red corpuscles, the presence of blood cells in the tissues, the destruction of groups of cells by toxins, bacteria, and parasites, certain split products of proteins, etc., may give to monocytes and macrophages the food required for their proliferation and bring about monocytosis, as well as local accumulation of macrophages in the tissues. Like amebæ, macrophages utilize for their nutrition the nitrogenous particles that they phagocyte. The various substances taken up by the cells are elaborated in their rhagiocrin apparatus.¹⁰ The neutral red vesicles increase in size and segregate a number of granules, which disappear if the colonies are starved. The substances manufactured by the rhagiocrin organs are probably used by the cell itself, or set free in the medium. Some of the products of the macrophages consist of substances which promote the multiplication of epithelial cells and fibroblasts¹¹ and are analogous to those present in embryo juice. Monocytes may secrete these substances in the culture medium¹² and stimulate the growth of colonies of fibroblasts.⁶ They transform proteins from serum and from the nitrogenous material of living or dead cells into food which they may bring to fixed tissue cells.¹¹ The nature of their secretions depends

¹⁰ Renaut, J., *Arch. anat. micr.*, 1906-07, ix, 495.

¹¹ Carrel, A., *J. Exp. Med.*, 1922, xxxvi, 385.

¹² Carrel, A., and Ebeling, A. H., *J. Exp. Med.*, 1922, xxxvi, 645.

(Figs. 3, 6, and 7). At the same time, the mitochondria augment in length and number. The segregation apparatus of the macrophage is far larger than that of the fibroblast on account of the greater metabolic activity of the former cell. When macrophages are fed on certain split products of proteins, or on fragments of dead muscle, they multiply more actively and develop more numerous and larger neutral red vesicles which generally contain small granules. If they are starved in Tyrode or Ringer solution, the neutral red vesicles lose their granules, decrease in size and number, and the cells themselves become smaller. In the macrophage, as in the fibroblast, the development of the segregation apparatus and of the mitochondria depends on the composition of the pericellular fluid.

The ability of the macrophage to multiply when fed upon blood serum⁶ or particles of proteins, and the fact that it dies in pure embryo juice, sharply distinguish this cell from the fibroblast. The fibroblast stops multiplying in blood serum and does not phagocytose other cells and fragments of proteins. It may feed upon substances set free in the medium by leucocytes⁶ and thyroid gland, and proliferate indefinitely in plasma and embryonic juice. A similar phenomenon occurs with proteose which, at a certain concentration, promotes the growth of fibroblasts and kills the macrophages.⁷ A third property distinguishing the macrophage from the fibroblast is a different susceptibility of the cells to arsenous oxide. The fibroblast dies in a concentration of arsenous oxide which is completely innocuous for the macrophage.⁸ The causes and the mechanism of the contrasting characteristics of the two types of connective tissue cells are still unknown.

It is certain that the innate physiological properties of the macrophages determine their behavior within the organism. The knowledge of these fundamental characteristics necessarily throws some light on the mechanism of the phenomena in which the cells participate. Macrophages are rarely present in the blood,⁹ and monocytes are not

⁶ Carrel, A., and Ebeling, A. H., *J. Exp. Med.*, 1923, xxxviii, 513.

⁷ Carrel, A., *Compt. rend. Soc. biol.*, 1926, xciv, 1060. Baker, L. E., and Carrel, A., *J. Exp. Med.*, 1926, xlv, 387, 397.

⁸ Carrel, A., unpublished experiments.

⁹ Rowley, M. W., *J. Exp. Med.*, 1908, x, 78. Simpson, M. J., *J. Med. Research*, 1922, xliii, 77.

tissues. Their dual and opposite rôles in the war on, and the propagation of, disease are accessory phenomena.

This study of the biological characteristics of the fibroblast and the macrophage is still very incomplete. However, the methods by which this important aspect of cytology may profitably be investigated have been built up and can be widely used. They will give rise to a thorough knowledge of the fundamental properties of both types of connective tissue cells, and ultimately reveal the mechanism of the normal and pathological phenomena in which these cells are involved.

CONCLUSIONS.

1. Monocytes and tissue macrophages become identical in appearance when they live under identical conditions. Macrophages cultivated in nutrient and non-nutrient media acquire different structures. Monocytes and tissue macrophages are mere functional variations of a single type.

2. The structure of the segregation apparatus and of the mitochondria of monocytes and macrophages depends on the composition of the pericellular fluid and on the metabolic state of the cells.

3. The monocyte and the macrophage are endowed with a number of physiological properties which become apparent when they grow in pure cultures.

4. The knowledge of these fundamental characteristics explains the behavior of the cells within the organism.

EXPLANATION OF PLATES.

The yellow tone in the original drawings is omitted in reproduction.

PLATE 8.

FIG. 1. Culture 8740-D7. Polymorphonuclear leucocyte after 2 hours cultivation in plasma and embryo juice.

FIG. 2. Culture 8740-D7. Red blood corpuscle.

FIG. 3. Culture 8740-D7. Blood monocyte after 2 hours cultivation in plasma and embryo juice.

FIG. 4. Culture 8741-D1. Blood monocyte after 2 hours cultivation in plasma and embryo juice.

FIG. 5. Culture 8740-D6. Blood monocyte after 24 hours cultivation in plasma and embryo juice.

possibly upon the composition of the substances they ingest. It is probable that the products of macrophages fed upon muscle fibers or bacteria are not identical. Antibodies may be a mere by-product of the nutrition of the macrophage. However, it is not known in what measure the cells use bacterial proteins for their own nutrition or even whether they use them at all. They can simultaneously secrete natural hemolysin and growth-promoting substances for epithelial cells and fibroblasts,¹³ oppose a foreign invader,¹⁴ and stimulate the tissues of the organism. The secretion of growth-promoting substances may explain the resumption of the activity of epithelial cells and fibroblasts in cicatrization, regeneration, sclerosis of organs, and tumor growth.¹¹ However, proliferation of resting fibroblasts and epithelial cells can also be determined by certain split products of the protein molecule.⁷ The proliferation of cells within the inhibiting humors of the adult organism may be due to the production of proteoses by degenerating material as well as to the setting free in the tissues of proteins of the embryonic type. The secretion of ferments is not observed to take place in pure cultures of macrophages. The macrocytase of Metchnikoff remains inside of the cells or, if it is set free, the conditions of the medium do not allow any manifestation of its activity. When macrophages are transformed into malignant cells by Rous virus,¹⁵ they digest the fibrin of the medium and give to the coagulum its characteristic appearance. Proteolysis only occurs around sick or dead macrophages.

There is no doubt that the function of the macrophages is far more important than was thought by Metchnikoff. In some cases, they are the defenders of the organism against infections, but they may also be the direct source of the invasion of the tissues by a disease such as Rous sarcoma.¹⁶ The destruction by phagocytosis of cells and bacteria, the secretion of antibodies, the reproduction of Rous virus, and the genesis of sarcoma cells, are phenomena of secondary importance,—a pathological adaptation of general physiological properties. The main function of the macrophages is related to the nutrition of the

¹³ Carrel, A., *J. Am. Med. Assn.*, 1924, lxxxii, 255.

¹⁴ Gay, F. P., and Morrison, L. F., *J. Infect. Dis.*, 1923, xxxiii, 338.

¹⁵ Carrel, A., *J. Am. Med. Assn.*, 1925, lxxxiv, 157.

¹⁶ Carrel, A., *Paris. méd.*, 1926, lix/lx, 274.

FIG. 6. Culture 8740-D14. Blood monocyte after 48 hours cultivation in plasma and embryo juice.

FIG. 7. Culture 8740-D15. Blood monocyte after 72 hours cultivation in plasma and embryo juice.

FIG. 8. Culture 8740-D19. Blood monocyte after 96 hours cultivation in plasma and embryo juice.

FIG. 9. Culture 3777-C. Tissue macrophage after 24 hours cultivation in plasma and embryo juice.

FIG. 10. Culture 3790-C. Tissue macrophage after 24 hours cultivation in plasma and embryo juice.

PLATE 9.

FIG. 11. Culture 3386-C1. Blood monocyte cultivated in Tyrode solution for 4 days.

FIG. 12. Culture 3386-C2. Blood monocyte cultivated in serum for 4 days.

FIG. 13. Culture 3445-C1. Blood monocyte cultivated in Ringer solution for 4 days.

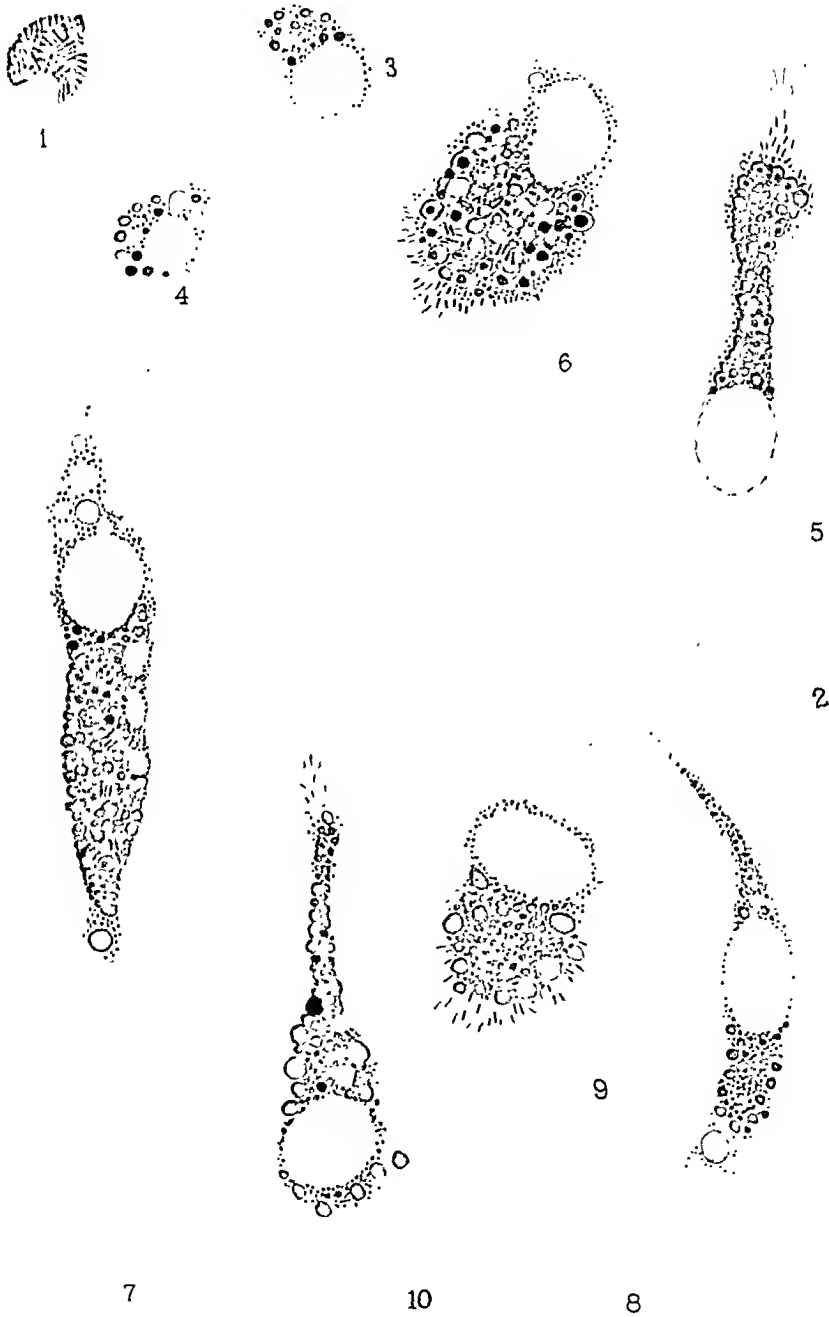
FIG. 14. Culture 3445-C2. Blood monocyte cultivated in serum for 4 days.

FIG. 15. Culture 3471-C1. Blood monocyte cultivated in Ringer solution for 3 days.

FIG. 16. Culture 3471-C2. Blood monocyte cultivated in serum for 3 days.

FIG. 17. Culture 3841-C1. Macrophage from subcutaneous tissue of an adult chicken after 24 hours cultivation in plasma and embryo juice.

FIG. 18. Culture 3841-C2. Macrophage from subcutaneous tissue of an adult chicken after 5 days cultivation in Tyrode solution.



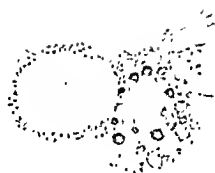
EBELING



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Plate 9

The search for pathogenic varieties of herpetomonads among the flagellates so commonly found inhabiting the intestinal tract of insects was begun in 1913 by Laveran and Franchini, who reported having induced in rats and mice a disease not unlike kala-azar or oriental sore by inoculation of flagellates obtained from the dog flea, mosquito, sheep ked, and house fly, and even of flagellates of plant origin.⁵ Pure cultures were used for inoculation in most instances. Later they obtained somewhat similar results with cultures of a flagellate obtained from the sand fly.⁶ Fantham and Porter⁷ induced symptoms of kala-azar by inoculation of various insect flagellates and believed that the leishmaniasis would prove to be insect-borne herpetomoniasis, but Hoare⁸ repeated their experiments and obtained only negative results. Becker⁹ and Drbohlav¹⁰ also failed to infect animals with flagellates of insect origin. Strong,¹¹ who found flagellate infections in the hemipteran, *Chariesterus cuspidatus*, of Panama, in its plant hosts, and in a lizard presumed to prey upon the insect, was able to cause ulceration of the skin in a monkey by inoculation of the flagellate material from the intestinal tract of the lizard and to demonstrate leishmaniform parasites in the skin lesion; but he found the plant and insect flagellates to be non-infective.

While a very large number of the plant and insect flagellates have been described, the greater part have been studied only under natural conditions and on the basis of morphological features, and they have usually been given specific names designating their hosts. It has been recognized, however, that the same flagellate may live in several hosts, and Becker,⁹ having demonstrated by cross-infection experiments that the flagellates of *Musca domestica* could thrive equally well in the intestinal tract of several other species of flies, and that the flagellates inhabiting each of the fly species which he studied showed a similar adaptability, concluded that the herpetomonads of flies all represent the same species. Drbohlav¹⁰ confirmed Becker's experiments with fly flagellates, using a cultivated strain of *Herpetomonas muscarum*, but was unable to infect the dog flea or water strider with any flagellate save the one naturally occurring in these insects. He was unsuccessful in establishing *Leishmania infantum* in any flagellate host studied.

A number of the flagellates of invertebrates and plants have been cultivated, among them *Herpetomonas culicis* from *Culex pipiens* (Novy, MacNeal,

⁵ Laveran, A., and Franchini, G., *Compt. rend. Acad.*, 1913, clvii, 744; *Bull. Soc. path. exot.*, 1914, vii, 665; 1919, xii, 379; 1920, xiii, 796.

⁶ Laveran, A., and Franchini, G., *Bull. Soc. path. exot.*, 1920, xiii, 569.

⁷ Fantham, H. B., and Porter, A., *Proc. Cambridge Phil. Soc.*, 1915, xviii, 39.

⁸ Hoare, C. A., *Parasitology*, 1921, xiii, 67.

⁹ Becker, E. R., *J. Parasitol.*, 1923, x, 25.

¹⁰ Drbohlav, J. J., *Am. J. Hyg.*, 1925, v, 580, 599.

¹¹ Strong, R. P., *Am. J. Trop. Med.*, 1924, iv, 1.

COMPARATIVE STUDIES OF HERPETOMONADS AND LEISHMANIAS.

I. CULTIVATION OF HERPETOMONADS FROM INSECTS AND PLANTS.

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PLATES 10 TO 15.

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Our interest in the herpetomonads parasitic in insects and plants in various parts of the world lies in the possible relationship of some of them to the parasites of kala-azar, of India, China, and Mediterranean countries, oriental sore, of Palestine, and espundia, of South America. The mode of infection of man by the leishmanias is not understood, and experimental work on the problem of transmission has, until recently,¹ been rendered extremely difficult by the lack of susceptible laboratory animals. The geographic distribution and epidemiology of the various forms of leishmaniasis have indicated that the parasites may be carried by some insect, and many attempts have been made to find evidence of this mode of transmission.

That the leishmanias may be taken up by insects and are capable of surviving in the insect gut was first shown in the case of *Leishmania donovani* by Patton,² who fed bedbugs on kala-azar patients and observed the development of the parasites to the flagellate stage in the bugs. Wenyon³ later reported the ingestion of *Leishmania tropica* from open lesions of cutaneous leishmaniasis by flies, mosquitoes, and bedbugs, and the development of the parasites in some of these insects. The sand fly (*Phlebotomus*) is looked upon at present⁴ as the most probable vector of leishmaniasis, but the experimental evidence is fragmentary.

¹ Young, C. W., and Liu, Pao-Yung, *Proc. Soc. Exp. Biol. and Med.*, 1926, xxiii, 392.

² Patton, W. S., *Scient. Mem. Off. Med. and San. Dept. Gov. India*, No. 27, 1907.

³ Wenyon, C. M., *Parasitology*, 1911, iv, 273.

⁴ Sinton, J. A., *Indian J. Med. Research*, 1924-25, xii, 701.

tus, *Culex pipiens*, *Musca domestica*, bluebottle flies (two strains), *Asclepias syriaca* (two strains), and *Asclepias nivea*. In addition, two impure cultures were obtained, one from *Oncopeltus cingulifer* (Fig. 4), and one from the plant on which it feeds, *Asclepias curassavica*. Attempts to purify these two strains were unsuccessful, and they soon died out.

The specimens of *O. cingulifer* were collected in Tela, Honduras,²⁰ those of *Oncopeltus sp.?* in the vicinity of Lima, Peru,²² those of *O. fasciatus* and *L. kalmii* on Long Island and in Shandaken, N. Y., those of *Anopheles quadrimaculatus* in Leesburg, Georgia,²³ and those of *Culex pipiens* near Jersey City. The house and bluebottle flies were collected on the grounds of The Rockefeller Institute. The specimens of *A. curassavica* were brought from Tela,²⁰ Honduras, and those of *A. syriaca* from Baltimore, Long Island, and Shandaken. For the Haitian milkweeds, *A. nivea*, we are indebted to Dr. Francis O. Holmes, of the Boyce Thompson Institute for Plant Research, Yonkers, N. Y., who infected them by allowing infected specimens of *Oncopeltus fasciatus* to feed upon the seed pods. For identification of the latex-feeding insects we are indebted to Dr. H. G. Barber, of the American Museum of Natural History. The bluebottle flies were no doubt *Calliphora*, but identification of the species was not possible in these instances.

Method of Cultivation.

For the initial cultivation of the flagellates from insects, solid blood agar plates were employed which contained various carbohydrates and were adjusted to different hydrogen ion concentrations, pH 5, 5.5, 6, 6.5, 7, and 7.5. The proportions of the constituents were as follows:

Defibrinated horse or rabbit blood.....	300 cc.
2 per cent nutrient agar, melted.....	500 cc.
Mixed sugar solution, sterilized by filtration through Berkefeld filters:.....	25 cc.
Glucose.....	20 gm.
Saccharose.....	10 "
Inulin.....	5 "

²² These specimens were collected by Dr. T. S. Battistini, of Lima.

²³ Dr. Mark F. Boyd, of the International Health Board, was kind enough to inform us of the presence of flagellates in these mosquitoes and also to dissect insects and inoculate culture media.

and Torrey¹²), *H. ctenocephali* of the dog flea (Tyzzer and Walker,¹³ Wenyon,¹⁴ Laveran and Franchini⁵), *H. pulicis* from *Pulex irritans* (Wenyon¹⁵), *H. clenopsyllæ*⁵ of the mouse flea, *H. phlebotomi*⁶ of the sand fly, *H. jaculum*⁵ of the scorpion, *Critidia melophagi*⁵ of the sheep ked, and *H. tarantolæ*¹⁶ of the gecko (Laveran and Franchini). Laveran and Franchini obtained pure cultures also from plants.¹⁷ Nöller¹⁸ cultivated a number of insect herpetomonads and several trypanosomes. Drbohlav¹⁰ obtained pure line strains of *H. muscarum*. The medium used has usually been the N. N. N. medium or some modification of it, though Franchini and Laveran made use also of blood bouillon,¹⁶ and Franchini¹⁹ later found that a number of flagellates would grow in a mixture of bouillon and plant latex.

The purpose of the present investigation was to cultivate a number of herpetomonads from different sources and compare their cultural and biological properties. Serological comparison of the leishmanias²⁰ had already indicated the value of immunological properties for differentiation and had suggested that these and other biological properties might be used for comparative studies of the leishmanias with other herpetomonads. We have been able to supplement this study by comparison of our strains with other cultivated flagellates, *H. ctenocephali*, isolated by Tyzzer and Walker in 1919,¹³ and *T. rotatorium*, isolated by Drbohlav,¹⁰ cultures of which were furnished us through the courtesy of Dr. E. E. Tyzzer, of Harvard University Medical School.

Pure cultures of herpetomonads have been isolated from the following insects and plants: *Oncopeltus fasciatus* (Fig. 1), *Oncopeltus sp.*?²¹ (Fig. 2), *Lygæus kalmii* (Fig. 3) (two strains), *Anopheles quadrimaculatus*

¹² Novy, F. G., MacNeal, W. J., and Torrey, H. N., *J. Infect. Dis.*, 1907, iv, 223.

¹³ Tyzzer, E. E., and Walker, E. L., *J. Med. Research.*, 1919, xl, 129.

¹⁴ Wenyon, C. M., *Tr. Soc. Trop. Med. and Hyg.*, 1914, vii, 104.

¹⁵ Wenyon, C. M., *Arch. Protistenk.*, 1913, xxxi, 1.

¹⁶ Laveran, A., and Franchini, G., *Bull. Soc. path. exot.*, 1921, xiv, 323.

¹⁷ Laveran, A., and Franchini, G., *Bull. Soc. path. exot.*, 1920, xiii, 796.

¹⁸ Nöller, W., *Arch. Schiffs- u. Tropen-Hyg.*, 1917, xxi, 53.

¹⁹ Franchini, G., *Bull. Soc. path. exot.*, 1923, xvi, 41.

²⁰ Noguchi, H., *Proc. Internat. Conf. Health Prob. Trop. America*, Kingston, Jamaica, British West Indies, July 22 to August 1, 1924, published in Boston, 1924, p. 455.

²¹ Specimens of this insect have recently been submitted for identification to Dr. H. G. Barber of the American Museum of Natural History, who was able at once to classify it as to genus. The species has not yet been determined.

stance an impure culture of flagellates from *A. curassavica* was obtained on a medium composed of the usual leptospira medium plus glucose, inulin, and tapioca.

The isolation experiments were carried out at room temperature, which varied from 18°C. to 28°C. during the day and was probably somewhat lower at night. All the plates were sealed with adhesive tape and kept in a moist chamber. Examinations were not made until after 7 to 10 days, experience having soon shown that there was no advantage in earlier examination. The colonies of flagellates develop very slowly, and they are so minute that very close examination is necessary to find them. In initial cultures 10 to 14 days were required in most instances for the colonies to become visible as tiny dew-like spots. When associated with bacteria, the flagellates can only be detected by microscopic examination, and for this purpose the dark-field microscope offers the most convenient and rapid means of detection.

From the pure colonies of flagellates found on plates with well separated colonies, subcultures were readily obtained on similar media (plates or slants), and still more certainly on leptospira medium, with or without carbohydrates. On this medium growth is remarkably rapid and prolific, in marked contrast to that of the first generation. All the strains isolated during the present experiments grow much more rapidly and profusely than any of the leishmanias or the strains of flagellates from dog flea or frog. They grow well at 37°C., as well as 25°C., while for the other flagellates the lower temperature is more suitable.

Description of Flagellates Studied.

Seven of the twelve strains isolated were derived from milkweed-feeding insects and their plant hosts. When the cultures came to be studied with respect to their serological and carbohydrate-fermenting properties, it was found that the seven strains represented in reality only two species of flagellates. Similarly, the two strains from mosquitoes behaved as one serologically. If we were to classify the strains according to host species, we should have to create several names for organisms which by morphology and biological reactions show identical characteristics, while if we were to depend on morphology alone, a number of flagellates which behave differently would have to be classed as a single species. The study of biological relationships, however, made it possible to identify a given flagellate in different environments and to separate different flagellates occurring in the same host. The classification of the strains was accordingly very much simplified. In order to avoid confusion it seems desirable to employ throughout this paper the nomenclature finally adopted,

Levulose.....	5 gm.
, Raffinose.....	5 "
Distilled water....	100 cc.
0.9 per cent sodium chloride solution.....	175 cc.

Various bacteria, molds, and yeasts are usually associated with the flagellates in the insect gut and by their more rapid growth render the isolation of the latter extremely difficult. In several instances impure cultures were obtained by inoculating plates with ascending dilutions of the suspension of teased viscera, and these were purified by transfers to new plates repeatedly until some pure colonies of flagellates appeared. Later, however, in the work with flies, it was found necessary to resort to Barber's technique²⁴ of picking out one flagellate or a group of them for inoculation of the plates. Theoretically this procedure ought to give fairly constant results, but actually many plates implanted with a single flagellate failed to show any growth, though plates spread gently with several organisms usually yielded some isolated colonies. Probably not every individual was capable of adapting itself to the cultural conditions provided.

It was noticed that whenever an impure culture was obtained by the plate method, the contaminating organisms were usually yeasts, which had apparently suppressed the growth of bacteria to some extent by the production of acidity. The observation suggested the use of culture media adjusted to concentrations of acid which were not sufficient to interfere with the growth of the flagellates, but in which the growth of bacteria was reduced so far as possible. The neutral and slightly alkaline media were not omitted in routine work, however. As a rule, four or five plates of a given hydrogen ion concentration were used; a small drop of the suspension of teased viscera in 0.5 per cent saline was spread on the first plate with a sterile bent pipette, and the other plates were brushed successively with the same pipette. The first plate was usually overgrown by bacteria, but one of the others might yield fairly discrete colonies of bacteria and an occasional pure colony of flagellates.

The cultivation of plant flagellates was much more difficult, not because of the contaminants—for in this instance the only contaminants present are the yeasts and molds from the stem or leaves—but because of the difficulty of adaptation of the flagellates to the media, as shown by the fact that when large numbers of them were introduced they usually lost their motility within a few days and finally degenerated. Pure cultures have thus far been obtained only on *leptospira* medium,²⁵ and only three times among many attempts. In one other in-

²⁴ Barber, M. A., *Philippine J. Sc., Section B*, 1914, ix, 307.

²⁵ The formula is as follows: 0.9 per cent NaCl, 800 parts; fresh rabbit serum, 100 parts; 2 per cent nutrient agar (pH 6.5 to 7.0), 100 parts; rabbit hemoglobin (made by laking 1 part of defibrinated blood with 3 parts of distilled water), 10 parts.

although the manner in which it was arrived at is chiefly discussed in Part II of our report.²⁶

Table I is a résumé of the names, source, and morphology of all the flagellates grown in pure culture. Identical strains from two or more sources have been named in accordance with the source from which they were first cultivated (*H. oncopelti*, *H. lygæorum*).²⁷ The mosquito flagellates, which proved identical, have been called *Herpetomonas culicidarum*, since the same strain was isolated from two genera of the family *Culicidæ*. As will be clear from the experiments described in Part II of this report, it was not possible to identify this organism with others previously described as occurring in mosquitoes.

The naming of the fly strains was a more difficult problem, inasmuch as each of the three proved to be distinct from the others. The preparations of the flagellates found in the gut of *Musca domestica* show chiefly large forms with double flagellum which conform with the descriptions of *H. muscæ domesticæ*, but the organisms seen in young cultures are not unlike *H. oncopelti* and *H. lygæorum*. As the cultures grow older much larger forms appear, but these do not correspond exactly with the naturally occurring individuals, and it is difficult to exclude the possibility that some other strain than *H. muscæ domesticæ* was simultaneously present in the insect and grew more readily in culture. It was likewise impossible to identify two distinct strains from *Calliphora* as *H. calliphoræ*. The strains were finally given the new species designations, *Herpetomonas muscidarum*, *H. media*, and *H. parva*.

All the flagellates cultivated have a single anterior flagellum, arising near the parabasal body, which is situated beside, or in front of, the nucleus. No undulating membrane, such as characterizes *Critithidia* and *Trypanosoma*, has been demonstrated in any of the strains, either under natural conditions or in culture. The term *Leptomonas*, while it has priority over *Herpetomonas*, is less well defined, and it seems reasonable, as suggested by Wenyon,¹⁵ to restrict the name to the species for which it was created until it can be proved that *Herpetomonas* is actually a synonym of it. Werner's objection to *Herpeto-*

²⁶ Noguchi, H., *J. Exp. Med.*, 1926, xlv, 327.

²⁷ The name *H. lygæi* has been used by Patton (*Arch. Protistenk.*, 1909, xiii, 1) to designate a flagellate species found in *Lygæus militaris* of India.

TABLE I.

Species.	Source.	Morphology in insect or plant.	Morphology in culture on <i>Leptospira</i> medium.
<i>H. oncopelti</i> .	<i>Oncopeltus fasciatus</i> .	Narrow, slender bodies; anterior end rounded, posterior pointed. Flagellum about the length of the body.	
	<i>Oncopeltus</i> sp.? <i>Lygus kalmii</i> No. 1.	Characteristic ribbon-like bodies, sometimes twisted; flagellum usually short.	
	<i>Asclepias syriaca</i> No. 213. <i>Asclepias nitida</i> .	Slightly longer and narrower than flagellates of <i>L. kalmii</i> No. 1; flagellum somewhat longer.	Similar to insect forms, but usually smaller, with shorter flagellum. Anterior end sometimes truncated.
<i>H. lygaeorum</i> .	<i>Lygus kalmii</i> No. 2.	Characteristic ribbon-like forms, but somewhat shorter than those of <i>A. syriaca</i> No. 213.	
	<i>Asclepias syriaca</i> No. 46.	Short, truncated forms, with short flagellum.	Similar to forms in insect, but considerably larger.
<i>H. culicidarum</i> .	<i>Anopheles quadrimaculatus</i> <i>Culex pipiens</i> .	Large forms, many with characteristic long double flagellum.	Similar to <i>H. oncopelti</i> in young cultures; much larger in old cultures, but never as large as <i>H. musæ domestica</i> of von Prowazek.
	<i>Musca domestica</i> .	Smaller than flagellates of <i>M. domestica</i> ; flagellum long.	Similar to young cultures of <i>H. muscoidarum</i> , but smaller and narrower; never become large.
<i>H. media</i> .	<i>Calliphora</i> sp.? No. 1.	Similar to <i>H. media</i> .	Small, rounded forms, with very short flagellum.
<i>H. parva</i> .	<i>Calliphora</i> sp.? No. 2.		

hours at room temperature as a grayish white, usually homogeneous mass, which gradually extends deeper into the medium. At 37°C. growth can be recognized macroscopically after 24 hours. On blood agar slants grayish, dew-like, moist and shining colonies become visible within 72 hours at room temperature and 48 hours at 37°C. They gradually spread and become confluent, and the layer of growth assumes a light grayish color. The colonies are of a sticky consistency, and a thin grayish pellicle may form on the surface of the condensation water.

The morphological features vary greatly according to the medium on which the organism is grown. In young colonies on the surface of blood agar slants (Figs. 6,*a*, 10,*a*) most of the individuals are pear-shaped, oval, or truncated at the anterior end, with a short flagellum which moves constantly from side to side. The flagellum arises far in the interior of the cell, the parabasal body lying at one side of the nucleus. After some days the organisms become aggregated into rosettes of varying size, the flagella being directed inward, the bodies becoming uniformly narrower, pyriform, or spindle-shaped, and the flagella longer. In the condensation water much longer, slenderer forms are found, with flagella as long as the body.

The flagellates grown on leptospira medium are of elongate form, with flagella usually the length of the body (Figs. 6,*b*, 10,*b*). Unusually long forms are found in cultures which have been kept at room temperature more than 2 or 3 weeks, and these resemble the specimens of the organisms in the insect host. The parabasal body occupies a position anterior to the nucleus, the anterior portion of the organism is wide and rounded, and the body narrows down to a slender, pointed posterior portion. These long forms with well developed flagella travel about freely and swiftly in all directions, in contrast to the short truncated forms, with vibrating flagella, which rotate swiftly but do not travel out of the microscopic field.

Numerous bizarre forms appear in media having a pH near 5. In some the cytoplasm is divided except at the anterior portion, where a single flagellum arises, the division beginning near the anterior portion or at any point farther toward the posterior end. There may be one, two, or several clefts in a single organism, and the lobes are usually unequal in size. It is difficult to interpret this phenomenon, which

monas,²⁸ on the ground that it was created for a biflagellate genus, has been invalidated by Wenyon's cytological study¹⁵ of *H. muscæ domesticæ*, which demonstrates that the biflagellated forms are actually individuals in a very early stage of division.

Herpetomonas oncopelti, n. sp. (Figs. 5, 6, 9, 10, 13, 14, 17, 18).

Strain I.—This strain (Fig. 5) was obtained from the intestinal tract of *Oncopeltus fasciatus* (Fig. 1) caught in Shandaken, N. Y. The insect feeds on the latex of the common milkweed, *Asclepias syriaca*, which is of widespread occurrence in the temperate regions of the United States. According to Holmes,²⁹ the insect is rarely found north of New Jersey, being replaced in the northeastern states by the species *Lygæus kalmii*, which also feeds on the latex of *A. syriaca*. Only three specimens of *O. fasciatus* have been obtained in the course of the present study (one on Long Island, and two in Shandaken), and only one of these was infected with herpetomonads. Farther south, however, in New Jersey and Maryland, Holmes found that the majority of specimens harbored flagellates.

Morphology in Insect Host.—The forms found in the insect (Figs. 5, 9, 17) have relatively narrow bodies, the anterior end is somewhat rounded, and the body gradually narrows to a point at the posterior end. The flagellum originates near the parabasal body, which is round or oval and is situated closer to the anterior end than to the nucleus. In most specimens the flagella are about the length of the body, perhaps longer. When the organisms are moving swiftly in forward motion, the flagellum vibrates rapidly; in less active specimens it has an undulating, serpentine motion and may sometimes be alternately coiled into loops and stretched out. None of the flattened or twisted ribbon-like bodies so characteristic of plant herpetomonads has been encountered. Occasionally short, broad, pear-shaped forms have been observed, some of which have long and others very short flagella.

Cultural Properties.—Once obtained in culture, the organism is easily grown on the semisolid leptospira medium or on any of the blood agar slants, such as the N.N.N. or Nöller's medium, which have been widely employed for the cultivation of leishmanias and insect flagellates. On leptospira medium the growth becomes visible within 48

²⁸ Werner, H., *Arch. Protistenk.*, 1909, xiii, 19.

²⁹ Holmes, F. O., *Biol. Bull.*, 1925, xlix, 323.

kalmii. The preparations made from suspensions of the intestinal tract show that the forms in *Lygæus* No. 2 were a trifle narrower and longer than the majority of those in *Lygæus* No. 1, and had somewhat longer flagella. The morphological characteristics of the two cultures, however, are very much the same (Figs. 18, 20). The two species can be distinguished only by serological and fermentation reactions.

Strain 2 (from Asclepias syriaca No. 46).—Figs. 11, 12. The impure culture of this strain was discovered several weeks after inoculation of leptospira medium with the plant latex. Purification was accomplished by the Barber technique. In the plant (Fig. 11) and also in culture (Fig. 12), this organism is indistinguishable from the strain from *A. syriaca* No. 213, except for slight difference in size, but serologically and in fermenting properties it is identical with *H. lygæorum*.

Herpetomonas sp.? from Oncopeltus cingulifer (Figs. 7, 8, 33, 34).

Oncopeltus cingulifer (Fig. 4) feeds on the latex of *Asclepias curassavica*, a Honduran milkweed, in which flagellates were first found by Hegner.²¹ The relation between the insect strain and that of its plant host could not be ascertained in this instance, since pure cultures were not obtained. The impure growth was detected 75 days after inoculation on one of several dozen plates which had been similarly inoculated and kept under identical conditions. The flagellates of *O. cingulifer* differed in appearance from *H. oncopelti* in having a considerably longer flagellum.

Herpetomonas sp.? from Asclepias curassavica (Figs. 15, 16, 31, 32).

An impure culture of this organism was obtained on a semisolid (leptospira) medium containing glucose, inulin, and tapioca. The flagellates in the culture (Figs. 16, 32) bore rather slight resemblance to the forms found in the latex (Figs. 15, 31) and were much longer than those of the pure cultures isolated subsequently from American and Haitian milkweeds (Figs. 12, 14). Their morphological features do not correspond with those of the cultural forms (impure) of *H. elmassiani* obtained by Migone from *Aranjia angustifolia* in Paraguay, as described by França.³¹

Herpetomonas culicidarum, n. sp. (Figs. 21, 22).

Through the cooperation of Dr. Mark F. Boyd, of Leesburg, Georgia,²³ we were able to secure an impure culture of a *Herpetomonas* from *Anopheles quadrimaculatus* on a blood sugar slant (pH 5). The strain was purified by plating on similar medium. The original film preparation made by Dr. Boyd (Fig. 21) contained numerous flagellates of truncated and spindle shape, with short flagella. The flagellates in culture (Fig. 22) were considerably longer, in this instance, than those in the original material. In all other instances the reverse was true.

has also been observed under natural conditions in the insect host, but it appears to be an abnormal multiple division.

Strain 2 (from Oncopeltus sp.?).—Figs. 9, 10. Another species of *Oncopeltus* (Fig. 2) was obtained by Dr. Telémaco S. Battistini in the vicinity of Lima. Its plant host is also a species of *Asclepias*. (The specimens of milkweeds which Dr. Battistini collected, however, proved to be free from flagellates.) The initial impure culture of this flagellate was purified by the Barber method. No morphological differences could be detected between this strain and the one derived from *Oncopeltus fasciatus*.

Strain 3 (from Lygæus kalmii No. 1).—Figs. 17, 18. *Lygæus kalmii* (Fig. 3) resembles *O. fasciatus* in appearance, and its geographic distribution is somewhat similar. In the Catskill Mountains (Shandaken), at an elevation of 1000 feet above sea level, *Lygæus kalmii* far exceeded *O. fasciatus* in number. Four of the twenty-four specimens collected harbored flagellates, and in two instances cultivation was successful. While the two strains were morphologically indistinguishable in culture, they proved subsequently to be distinct species, one of which is identical with *H. oncopelti*.

Strain 4 (from Asclepias syriaca No. 213).—The occurrence of herpetomonads in *Asclepias syriaca* (Fig. 30) was first reported by Holmes,³⁰ who regarded the organisms found by him as identical with *H. elmassiani* of Migone, described by França.³¹ Of two strains of flagellates isolated from two infected milkweeds collected in Shandaken, N. Y., one proved to be *H. oncopelti*. The initial growth was obtained on leptospira medium and was not detected until several weeks after inoculation. The culture was purified by plating.

Strain 5 (from Asclepias nivea).—Figs. 13, 14. Through the kindness of Dr. Francis O. Holmes, of the Boyce Thompson Institute for Plant Research, a plant of the species *Asclepias nivea*, a Haitian milkweed, was placed at our disposal. It had been infected by allowing infected specimens of *Oncopeltus fasciatus* to feed on the seed pods. The morphology of these flagellates in the latex (Fig. 13) is similar to that of *H. oncopelti* in *Asclepias syriaca* (Fig. 30). They are characteristically ribbon-like, with twisted bodies, clear, almost hyaline protoplasm, and short flagellum. In culture (Fig. 14), however, they cannot be distinguished from the culture forms derived from hemipterans. The initial culture was pure, and, as in the case of all the plant strains, was obtained on leptospira medium. Only one tube of twelve inoculated yielded growth.

Herpetomonas lygæorum, n. sp. (Figs. 11, 12, 19, 20).

Strain 1 (from Lygæus kalmii No. 2).—As has already been mentioned, cultures of herpetomonads were obtained from two insects of the species *Lygæus*

³⁰ Holmes, F. O., *Phytopathology*, 1924, xiv, 146.

³¹ França, C., *Ann. Soc. belge med. trop.*, 1920-21, i, 245.

Strain from Culex pipiens.—Small numbers of herpetomonads were found in the intestines of two larvæ of *Culex pipiens*, collected in New Jersey, and from one of them a culture was obtained on acid blood agar plates. The immunological and fermenting properties of this strain are identical with those of the flagellate from *A. quadrimaculatus*.

Herpetomonas muscidarum, n. sp. (from *Musca domestica*) (Figs. 23, 24).

Many of the house flies collected about the grounds of The Rockefeller Institute harbored herpetomonads, some of them very large forms with long active flagellum, in many instances double, as described by von Prowazek.³² A section of intestinal tract from one of the infected flies, in which the flagellates were swarming as in culture, was picked out intact with a fine pipette, washed, and the contents suspended in 0.5 per cent saline. Inoculation of acid blood agar with ascending dilutions of this suspension yielded some pure colonies.

While the cultural properties of this organism are very much the same as those of the other cultivated strains of the series, in morphology it is slightly different. In young cultures on leptospira medium (Fig. 24, a) the individuals are no larger and the flagella no longer than in the case of *H. oncopelti* and *H. lygæorum*, but after several weeks in this medium extremely long forms appear, with flagella not unlike those of the organisms in the original material (Fig. 24, b).

Herpetomonas media, n. sp. (from *Calliphora* No. 1) (Figs. 25, 26).

This culture, which was obtained by the Barber method, is a pure line strain. Morphologically this organism resembles *H. muscidarum*, but it never attains the size of that strain, even in old cultures, and it is immunologically distinct.

Herpetomonas parva, n. sp. (from *Calliphora* No. 2) (Figs. 27, 28).

This culture also represents a pure line strain. It is decidedly smaller than the other two strains from flies. On blood agar slants the colonies of *media* and *parva* are light gray, heavier than those of *muscidarum*, and show less tendency to coalesce.

Herpetomonas ctenocephali; *Trypanosoma rotatorium*; the *Leishmanias*.

Morphologically, *H. ctenocephali* is quite unlike any of our strains. In its leaf-like form it resembles rather the flagellates found in the plant latices, though the flagellum is much longer. The frog trypanosome is again entirely different from any of the other flagellates studied. On the other hand, the morphological features of the leishmanias, while distinctive, approach those of the culture

³² von Prowazek, S., *Arb. k. Gsndtsamte*, 1904, xx, 440.

forms of our insect and plant flagellates; they are pyriform and stocky when grown on the surface of blood agar slants, but characteristically longer and slenderer on leptospira medium, and have longer flagella.

The flagellates of the dog flea and frog grow very slowly, 1 to 2 weeks being required for the growth on blood agar slants to become visible. The leishmanias grow somewhat more rapidly, but much more slowly than the plant and insect strains. All the strains studied show more rapid and prolific growth on leptospira medium than on plates or slants.

Table II is a comparison of certain morphological features of the flagellates studied. Preparations of cultures grown on leptospira medium were used in making the measurements of culture forms, except in the case of *Leishmania brasiliensis*; in this instance the stocky forms obtained from a blood slant culture were measured.

The preparations used as a basis of comparison were all made by fixing dried films in methyl alcohol and staining with Giemsa's solution. The early part of the work was not done under laboratory conditions, and this method was the only one which could be conveniently carried out. Later the cultures were studied by cytological methods (wet fixation in Schaudinn's sublimate alcohol, followed by staining with iron-hematoxylin or Giemsa's solution, with subsequent differentiation), but since these preparations furnished no additional information regarding the structure of the organisms and were not available in all instances, they have not been especially mentioned in the present study.

SUMMARY.

Nine strains of herpetomonads have been isolated in pure culture from eight varieties of insects, and three strains from two species of plants. Four of the cultures were derived from latex-feeding insects (*Oncopeltus fasciatus*, *Oncopeltus* sp. ?, *Lygæus kalmi*) and three from latex plants (*Asclepias syriaca*, *Asclepias nivea*); two from mosquitoes (*Culex pipiens* and *Anopheles quadrimaculatus*), one from the house fly (*Musca domestica*), and two from bluebottle flies. In addition impure cultures have been obtained from *Oncopeltus cingulifer* and from its plant host, *Asclepias curassavica*.

The flagellates cultivated, all of which belong to the genus *Herpetomonas*, have been identified chiefly by their biological relationships,

<i>Calliphora</i> sp.	16-20	1.5-2	2-2.5	2-2.2	6-16	2.5-3	8-25	Strain 1.
" " (culture forms)...	6-18	2-3	2-5	2-2.2	3-5	2.5-3	10-20	" 1.
" " " "	5-9	1.8-2	2-2.5	1.8-2.2	2-3	2-2.5	4-8	" 2.
<i>Asclepias syriaca</i>	8-12	1.8-2	1.8-2	2-2.2	4-6	2-2.5	2-10	Shandaken plant 46.
" "	13-22	1.5-2	1-2.5	2-3	5-14.5	2-3.5	6-12	" " 213.
" " (culture forms)...	6-12	1.8-3	0.5-2.5	1.8-2.5	3-4	2-2.2	4-14	Strain 46.
" " " "	7-17	1.5-3.5	2-2.4	1.8-2.5	1-6	2.8-3.5	6-16	" " 213.
<i>Asclepias nirea</i>	13-22	1.5-2	2-3	2-3	5-14.5	2-3.5	6-12	
" " (culture forms)...	10-17	2-4	2-4	2.5-3	2-6	2.5-3.5	6-18	
<i>Asclepias curassavica</i>	14-20	1.2-2	1.3-4	2-3	8-14	2-4	3-12	Honduras.
" " (culture forms)...	10-18	2-4	2-3	2-2.5	2-6	2.5-3	6-20	Impure culture.
<i>Ctenocephalus canis</i> .								
<i>H. ctenocephali</i> H.	Round, 3.3-6					2.5-4.3		After Tyzzer and Walker. ¹³
" " " "	Kinetic, 11-16.7					1.3-2.5	7.3-21	
" " (culture forms)	10-15	1-1.2	1-1.5	1.5-1.8	5-10	1.8-2	15-18	H strain. Twisted. Sharply pointed at both ends.
<i>Rana pipiens</i> .								
<i>Trypanosoma rotatorium</i> (culture forms).....	10-15	1-1.2	1-1.5	1.5-1.8	5-10	1.8-2	6-8	Twisted and sharply tapered posterior portion.
Oriental sore.								
<i>Leishmania tropica</i>	10-12	1.5-2	1.8-2.2	1.8-2.2	6-8	2-2.5	10-15	Slender forms.
Espundia.								
<i>L. brasiliensis</i>	5-8	1.8-2	0.5-1	1.8-2.2	1-2.5	1.5-3	3-6	Stocky "
Kala-azar (China).								
<i>L. infantum</i>	9-12.5	1-1.2	1-1.5	1.5-1.8	4-6	2-2.5	3-6	Sharply drawn to ends.

of the culture. The flagellates grown on the surface of blood slants are pyriform, with truncated anterior portion, and short flagellum; in the condensation water, however, the individuals are elongated and have long active flagella. On the leptospira medium the slender active forms with long flagella predominate. In the presence of fermentable carbohydrate, or in medium containing considerable acid, peculiar bifurcated or multifurcated individuals are seen. Similar forms have been seen under natural conditions. Cultures of *Leishmania* behave in the same way under the conditions described.

There is a striking difference in rapidity of growth between the organisms isolated by us and the leishmanias, *H. clenoccephali*, and *T. rotatorium*. While the stock cultures of the group first mentioned multiply rapidly at 37°C., growth becoming visible within 24 hours, the latter group grow scarcely at all at 37° and only slowly at 25°, 1 to 2 weeks being required for growth to become macroscopically demonstrable.

While the flagellum of the leishmanias, as also of *H. clenoccephali*, is long, serpentine in its movements, and heavy, having the appearance of being enveloped by a sheath throughout its entire length, that of the recently isolated strains is thin, less flexible, and without the sheath-like appearance. The only exceptions to this rule are the flagellates from *Musca domestica* and *Calliphora* No. 1, which have a long flagellum not unlike that of the leishmanias.

As the foregoing observations indicate, morphological differentiation of the flagellates studied, while not impossible, is subject to error by reason of the variations due to age and cultural conditions. The flagellates of the latex-feeding insects, the plants, the flies, and the mosquitoes can readily be distinguished from *Leishmania* by their rapid growth at 37°C., but their differentiation from one another is possible only by serological and fermentation reactions.

EXPLANATION OF PLATES.

PLATE 10.

FIGS. 1 to 4. Latex-feeding insects, natural size. Fig. 1, specimen of *Oncopeltus fasciatus* caught on Long Island. Fig. 2, *Oncopeltus* sp. from Lima, Peru. Fig. 3, *Lygæus kalmii*, specimen from Long Island. Fig. 4, *Oncopeltus cingulifer* from Honduras.

which will be described in detail in Part II of this report. The seven strains from latex-feeding insects and latex plants represent two distinct species, which have been designated *H. oncopelti* and *H. lygæorum*. The two strains from mosquitoes proved to be the same organism and have been called *Herpetomonas culicidarum*. The culture obtained from *Musca domestica* contained larger individuals than those of any other strain, and the organism is morphologically distinct from either of the *Calliphora* strains. None of the fly flagellates cultivated could be identified with the species *H. muscæ domesticæ* or *H. calliphoræ*, and hence they have been given new names, *Herpetomonas muscidarum*, *H. media*, and *H. parva*.

Blood agar plates were used for initial cultivation of the strains from insects and the semisolid leptospira medium for isolation of the plant flagellates. A number of the strains were purified by plating on acid blood agar, a procedure which reduces considerably the growth of bacterial contaminants. The Barber technique was utilized for isolation of the flagellates from flies, because of the very large number of bacteria found with them in these insects, and, in one or two instances, for the purification of impure cultures. Once they have been obtained in culture, all the strains grow well on leptospira medium, as well as on blood slants. Growth takes place both at 26°C. and at 37°.

The morphology of the organisms is considerably modified by cultivation. This is especially true of the plant flagellates. In the latex they have ribbon-like bodies, often twisted, and comparatively short flagella; the protoplasm is clear, almost hyaline. The flagellates seen in the gut and feces of insects are usually large, slender organisms, with flagella as long as or even longer than the body, which contains numerous volutin granules in the cytoplasm. In cultures under parallel conditions the flagellates from both these sources become shorter and thicker, the plant forms no longer appear flat and ribbon-like, and in general the organisms approach one another in morphological features. Even in the case of the least modified insect flagellates, *i.e.* those from flies, there is never exact correspondence between the natural and the cultivated forms.

The morphological features of the cultivated flagellates vary according to the medium on which the organisms are grown and the age

PLATES 11 TO 14.

FIGS. 5 to 28. Herpetomonads of insects and plants, as they appear under natural conditions and in culture. Drawn from preparations stained with Giemsa's solution after fixation by methyl alcohol. Magnification $\times 1200$. The species name and source are indicated in each instance. *a*, forms from slant cultures. *b*, forms from cultures on leptospira medium.

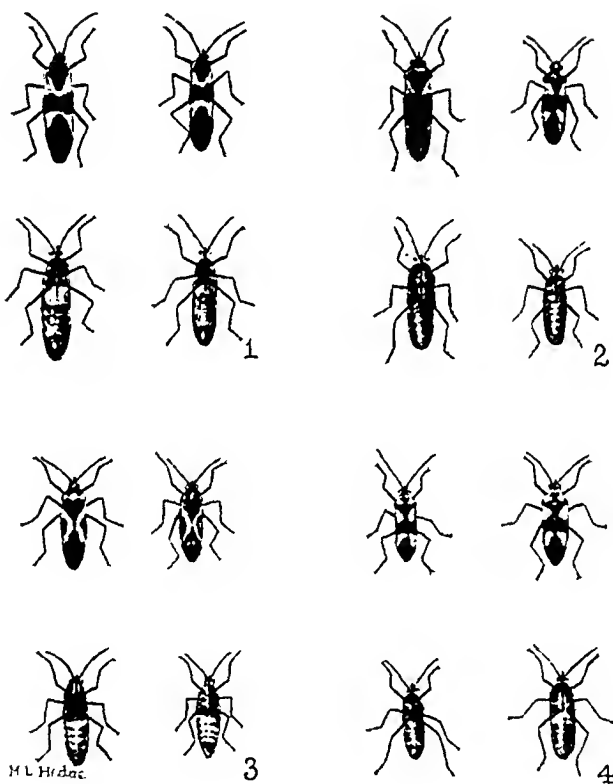
PLATE 15.

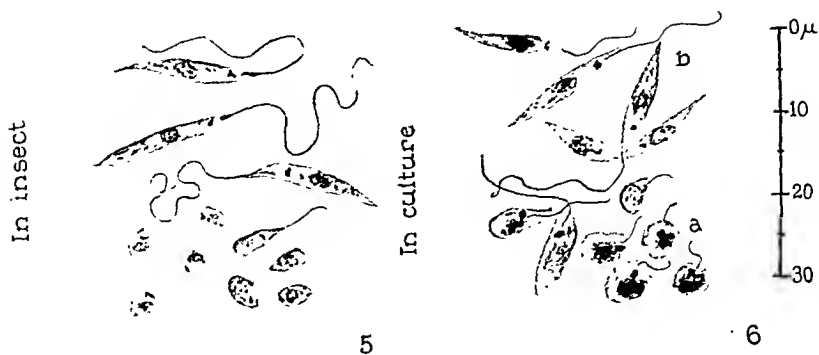
Magnification $\times 1200$.

FIG. 29. *Herpetomonas davidi*, original preparation made by Lafont,³³ presented by Professor Emile Brumpt. Giemsa's stain.

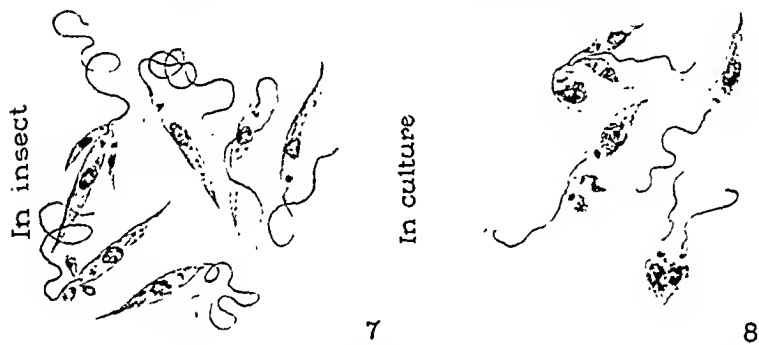
FIGS. 30 to 34. Drawings from preparations fixed in methyl alcohol and stained with Giemsa's solution. Fig. 30, flagellates from a specimen of *A. syriaca*, a plant of the group found by Holmes³⁰ in Maryland and studied by him. Fig. 31, flagellates from the latex of *Asclepias curassavica* (Honduras). Fig. 32, impure culture of flagellates from *Asclepias curassavica*. Fig. 33, flagellates from the gut of *Oncopeltus cingulifer*, which feeds on *Asclepias curassavica*. Fig. 34, impure culture of flagellates of *Oncopeltus cingulifer*.

³³ Lafont, A., *Compt. rend. Soc. biol.*, 1909, lxvi, 1011.

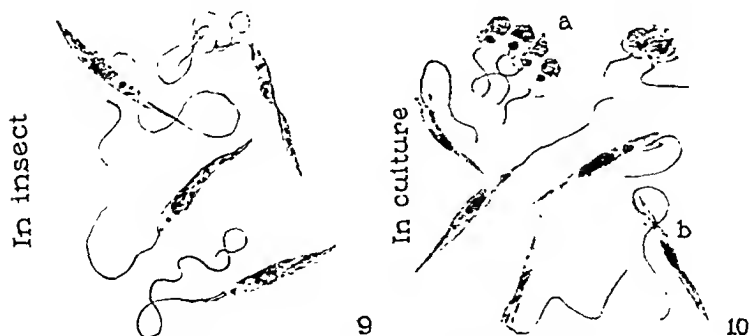




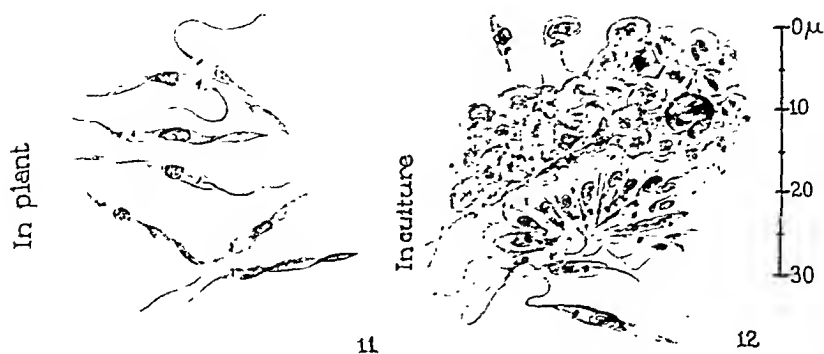
H. oncopelti (from *Oncopeltus fasciatus*)



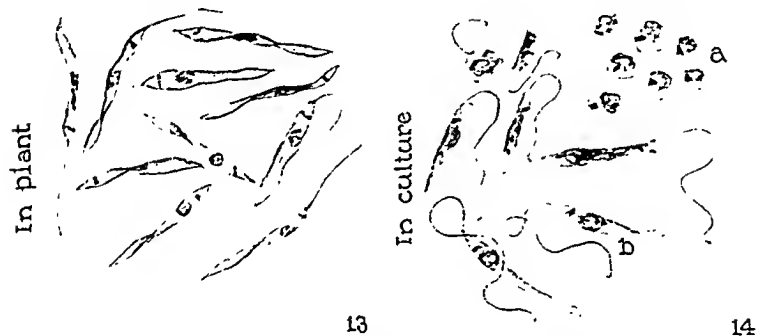
Herpetomonas sp.? (from *Oncopeltus cingulifer*)



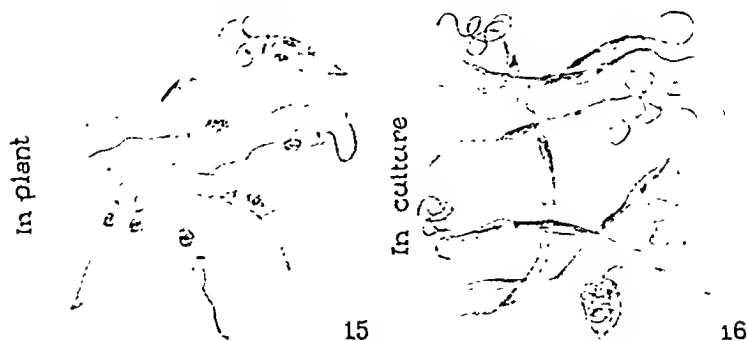
H. oncopelti (from *Oncopeltus* sp.?)



H. lygaeorum (from *Asclepias syriaca* No. 46)



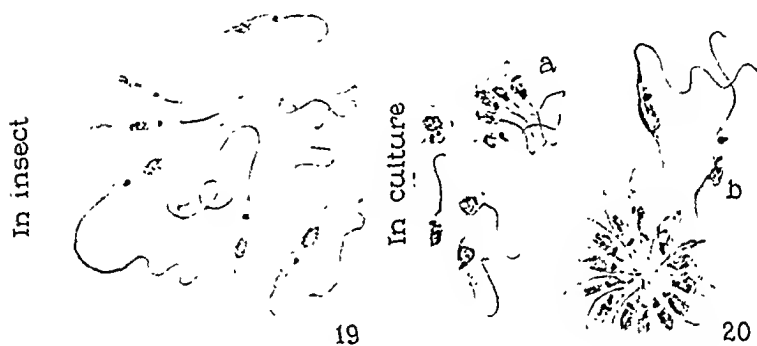
H. oncopelti (from *Asclepias nivea*)



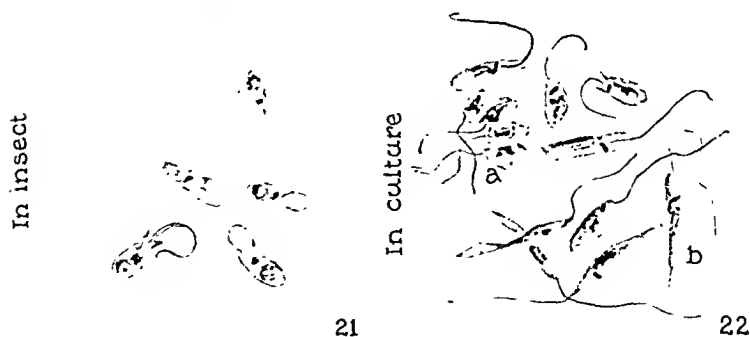
Herpetomonas sp.? (from *A. curassavica*)



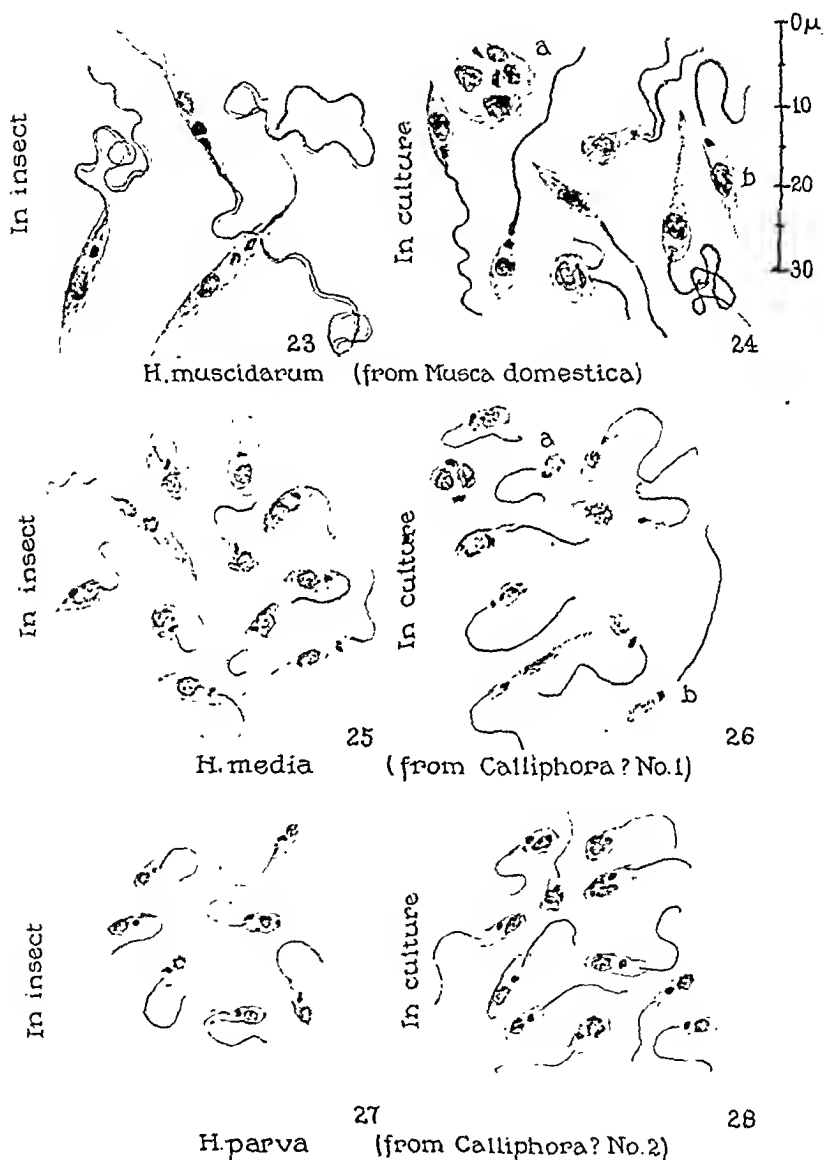
H. oncopelti (from *Lygaeus kalmii* No.1)



H. lygaeorum (from *Lygaeus kalmii* No.2)

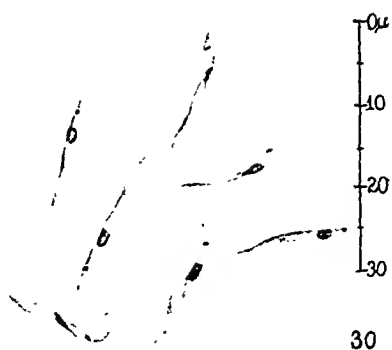


H. culicidarum (from *A. quadrimaculatus*)

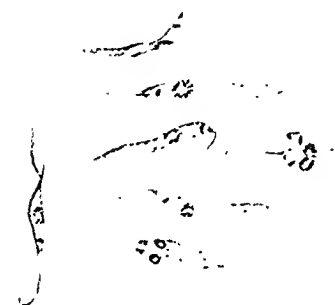




29



30



31



32



33



34

lated organisms; and the interpretation of the findings in such instances is complicated by the presence of natural flagellate infections in the animals employed (c.g., mice, rats, amphibians, and birds).

The immunological properties of the leishmanias and herpetomonads have been very little studied. The writer,⁴ in 1924, showed that at least three species of leishmanias could be distinguished by serological tests. Kligler⁵ has also found that *L. brasiliensis* and *L. infantum* are immunologically distinct from one another and from *L. tropica*, while different strains of *L. tropica* are serologically identical. Wagener and Koch⁶ recently carried out comparative serological tests with cultures of four strains of *Leishmania* and one strain of *Herpetomonas clenoccephali*, the latter a flagellate of the dog flea, which has been regarded as a possible vector of canine leishmaniasis. The anti-leishmania sera of these authors, which were prepared with killed cultures, had no effect upon *H. clenoccephali* but affected equally the different species of leishmanias.

Investigation of the chemical changes which may take place in various carbohydrate-containing culture media has not so far been utilized for the differentiation of species of *Herpetomonas*. Kligler⁵ attempted to distinguish leishmanias by this means but was unable to draw any definite conclusion from his study with a small series of strains.

As a first step in the application of immunological and biochemical methods to the differentiation of flagellate species, a number of strains of herpetomonads have been isolated from various sources and compared in serological and fermenting properties with one another and with the leishmanias. Twelve strains in all have been cultivated as described in Part I of this report.⁷ Two additional flagellate strains, *Herpetomonas clenoccephali* and *Trypanosoma rotatorium*, furnished by courtesy of Dr. E. E. Tyzzer, were included in the study. The leishmania strains were the same as those I have employed previ-

⁴ Noguchi, H., *Proc. Internat. Conf. Health Prob. Trop. America*, Kingston, Jamaica, British West Indies, July 22 to August 1, 1924, published in Boston, 1924, p. 455.

⁵ Kligler, I. J., *Tr. Roy. Soc. Med.*, 1925, xix, 330.

⁶ Wagener, E. H., and Koch, D. A., *Univ. Calif. Pub. Zool.*, 1926, xxviii, 365.

⁷ Noguchi, H., and Tilden, E. B., *J. Exp. Med.*, 1926, xlv, 307.

COMPARATIVE STUDIES OF HERPETOMONADS AND LEISHMANIAS.

II. DIFFERENTIATION OF THE ORGANISMS BY SEROLOGICAL REACTIONS AND FERMENTATION TESTS.

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Rogers, who first cultivated the parasite of kala-azar¹ and found that under cultural conditions it acquired a flagellum and became morphologically indistinguishable from the herpetomonads so widely distributed in nature as parasites of insects and plants, suggested that the organism might pass the flagellated stage of its life history in some blood-sucking insect.² The theory proved an extremely difficult one to substantiate, however. The extremely slow development of kala-azar and of other infections due to leishmanias, and the lack of susceptible animals, both contribute to the difficulty of carrying out transmission experiments. The fact that blood-sucking insects which have been allowed to feed upon patients with kala-azar or oriental sore are found on subsequent dissection to contain flagellates does not justify the conclusion that the flagellates are leishmanias, because the latter cannot be distinguished on morphological grounds from the flagellates occurring in many insects. Even when the insects used have been bred in the laboratory, and one may reasonably assume that they are free from flagellate infection, it is essential to be able to demonstrate in some way that the strain isolated from the insect after feeding is the same as that obtained from the patient. The injection into animals of herpetomonads derived from insects and plants³ has not given results which are sufficiently striking or constant to be considered convincing evidence of the pathogenicity of the inocu-

¹ Rogers, L., *Lancet*, 1904, lxxxii, 215.

² Rogers, L., *Proc. Roy. Soc. London, Series B*, 1906, lxxvii, 284.

³ Discussed in Part I of this report.⁷

TABLE I.

Agglutination Tests.

Source of flagellate.	Antisera.										
	Anti-oncopelti (strain from <i>O. fasciatus</i>).	Anti-tyzzerum (strain from <i>L. kalmii</i> No. 2).	Anti-cuticularum (strain from <i>Anopheles quadrimaculatus</i>).	Anti-muscicidarum.	Anti-media.	Anti-parva.	Anti-tropica.	Anti-brasilensis.	Anti-infantum.	Anti-donovani.	Normal rabbit serum.
<i>O. fasciatus</i>	++	+	-	-	-	-	-	-	-	-	-
<i>Oncopeltus</i> sp. ?.....	++	+	-	-	-	-	-	-	-	-	-
<i>L. kalmii</i> No. 1.....	++	+	-	-	-	-	-	-	-	-	-
" " 2.....	+	++	++	-	-	-	-	-	-	-	-
<i>A. quadrimaculatus</i>	-	-	++	++	-	-	-	-	-	-	-
<i>C. pipiens</i>	-	-	++	++	++	++	-	-	-	-	-
<i>M. domestica</i>	-	-	-	-	++	++	-	-	-	-	-
<i>Calliphora</i> sp.? No. 1.....	-	-	-	-	-	++	-	-	-	-	-
" " 2.....	-	-	-	-	-	-	-	-	-	-	-
<i>A. syriaca</i> No. 46.....	+	++	+	-	-	-	-	-	-	-	-
" " 213.....	++	++	-	-	-	-	-	-	-	-	-
" nivea.....	++	+	-	-	-	-	-	-	-	-	-
Oriental sore.	-	-	-	-	-	-	++	+	+	-	-
<i>L. tropica</i>	-	-	-	-	-	-	++	+	-	-	-
Espundia.	-	-	-	-	-	-	-	++	-	-	-
<i>L. brasiliensis</i>	-	-	-	-	-	-	-	++	++	++	-
Kala-azar.	-	-	-	-	-	-	+	++	++	++	-
<i>L. infantum</i>	-	-	-	-	-	-	-	-	++	++	-
" donovani.....	-	-	-	-	-	-	-	-	++	++	-
<i>Cletocephalus canis</i> .	-	-	-	-	-	-	-	-	-	-	-
<i>H. clenoccephali</i>	-	-	-	-	-	-	-	-	-	-	-
<i>Rana pipiens</i> .	-	-	-	-	-	-	-	-	-	-	-
<i>T. rotatorium</i>	-	-	-	-	-	-	-	-	-	-	-

++++, rapid, complete precipitation, agglutination involving both cytoplasm and flagella. +, definite effect on flagella, which are twisted and adhere to one another.

ously for immunological investigation.⁴ Comparison of the biological characteristics of these eighteen strains of flagellates brought to light a number of interesting facts.

Serological Differentiation of Herpetomonads and Leishmanias.

Rabbits were immunized against *Herpetomonas oncopelti*, *H. lygæorum*, *H. culicidarum*, *H. muscidarum*, *H. media*, *H. parva*, *Leishmania tropica*, *L. brasiliensis*, *L. infantum*, and *L. donovani* by intravenous injections of 1 to 2 cc. of rich living cultures at 4 day intervals on four successive occasions. The animals were bled 9 days after the last inoculation. The action of the specific immune sera thus prepared was tested upon the homologous and heterologous strains by agglutination and by complement fixation.

For the agglutination test, 0.05 cc. of immune serum was added to 1 cc. of a saline suspension of culture. A drop of the mixture was examined immediately with the dark-field microscope and again after 30 minutes and after 18 and 24 hours. Gross examination of the tubes was carried out as well. Control tests with normal rabbit serum were made in each instance. The presence in the suspension under test of rosettes or agglomerated masses did not interfere with the observations, since the changes brought about by specific agglutination are easily to be recognized. Under the dark-field microscope both flagellum and cytoplasm are seen to be profoundly affected by the specific immune serum. The organisms become sluggish or motionless, the bodies swollen and uneven in contour, the flagella twisted irregularly and adherent to whatever comes in contact with them, and finally the bodies are broken up. With a strong specific serum the gross findings are similarly striking. Within 30 minutes the turbid suspension becomes granular and begins to sediment and after 18 hours the supernatant fluid is clear, and there is a compact whitish sediment at the bottom of the tube.

For test of the specific complement-fixing power of the sera, the anticomplementary titer of a given antigen was first determined and half the anticomplementary dose employed. The immune serum was inactivated by heating at 55°C. for 30 minutes, and the amount used was 0.1 cc. in a total volume of 1 cc., 0.9 per cent saline being the diluent. The quantity of guinea pig serum, employed as complement, was 0.1 cc. of a 40 per cent dilution, and of anti-sheep amboceptor 2 hemolytic units. Incubation for fixation (30 minutes) and subsequent hemolysis (30 minutes) was carried out at 37°C.

The results of the agglutination and complement fixation tests are recorded in Tables I and II. The striking phenomena observed were as follows:

1. The strong reactions of the flagellates with their homologous sera. These were clear-cut and unmistakable.

2. The absolute indifference to anti-leishmania immune sera of the herpetomonad flagellates and of *T. rotatorium*, and *vice versa*, an indifference showing that no serological affinity exists between the leishmanias and the other flagellates studied.

3. The reciprocal reactions which took place among the different strains of herpetomonads derived from milkweeds and the insects feeding on the latex of these plants. For example, the anti-*oncopelti* serum had an equally strong effect upon cultures isolated from *Oncopeltus fasciatus*, *Lygæus kalmii* No. 1, the Peruvian species of *Oncopeltus*, *Asclepias syriaca* No. 213, and *Asclepias nivea*; while the anti-*lygæorum* serum had the same effect upon the culture derived from *Asclepias syriaca* No. 46 as upon the homologous strain. If these serological reactions may be regarded as indicating species specificity of herpetomonads, we may conclude that *Herpetomonas oncopelti* was present in three insects and two plants and *H. lygæorum* in one insect and one plant, or, viewing the matter from another point of view, that the insect, *Lygæus kalmii*, may harbor either *H. oncopelti* or *H. lygæorum*, as may also the plant, *Asclepias syriaca*.

4. The serological independence of three strains of herpetomonads derived from flies (a house fly and two bluebottle flies).

5. The serological identity of the two strains isolated from mosquitoes, one from *Anopheles* (adult) and the other from *Culex* (larva).

6. The inability of heterologous immune sera to affect *Herpetomonas clenoccephali* and *Trypanosoma rotatorium*.

The reactions among the leishmanias were similar to those previously reported, *L. tropica*, *L. brasiliensis*, and *L. donovani* being serologically distinct, and *L. infantum* related to *L. donovani*.

Fermentation of Carbohydrates by Herpetomonads and Leishmanias.

Leptospira medium, to which litmus had been added as an indicator of acid formation, and which contained the carbohydrate in a concentration of 1 per cent, was employed. All the flagellates grew luxuriantly on this medium at room temperature. The tubes were allowed to stand for 18 days before the final results were recorded, in order to permit the fermentation to reach the maximum point. Some of the sugars were split slowly. Table III shows the degree of acid formation, indicative of carbohydrate cleavage, observed in the various media.

TABLE II.

Complement Fixation Tests.

Source of flagellate.	Antisera.										
	Anti-oncopelti (strain from <i>O. fasciatus</i>).	Anti-lygaeum (strain from <i>L. kalmii</i> No. 2).	Anti-culicidarium (strain from <i>Anopheles quadrimaculatus</i>).	Anti-muscicidarium.	Anti-media.	Anti-porc.	Anti-tropica.	Anti-brasilensis.	Anti-infantum.	Anti-donorani.	Normal rabbit serum.
<i>O. fasciatus</i>	++	++	-	-	-	-	-	-	-	-	-
<i>Oncopeltus</i> sp. 1.....	++	++	-	-	-	-	-	-	-	-	-
<i>L. kalmii</i> No. 1.....	++	++	-	-	-	-	-	-	-	-	-
" " 2.....	++	++	-	-	-	-	-	-	-	-	-
<i>A. quadrimaculatus</i>	-	-	++++	++	-	-	-	-	-	-	-
<i>C. pipiens</i>	-	-	++++	++	-	-	-	-	-	-	-
<i>M. domestica</i>	-	-	-	-	++	++	-	-	-	-	-
<i>Calliphora</i> sp. No. 1.....	-	-	-	-	++	++	++	-	+	-	-
" " 2.....	-	-	-	-	-	-	-	-	-	-	-
<i>A. syriaca</i> No. 46.....	++	++	-	-	-	-	-	-	-	-	-
" " 213.....	++	++	-	-	-	-	-	-	-	-	-
" <i>nirca</i>	++	++	-	-	-	-	-	-	-	-	-
Oriental sore.	-	-	-	-	-	-	++	-	+	-	-
<i>L. tropica</i>	-	-	-	-	-	-	++	-	-	-	-
Espundia.	-	-	-	-	-	-	-	++	-	++	-
<i>L. brasiliensis</i>	-	-	-	-	-	-	-	++	++	++	-
Kala-azar.	-	-	-	-	-	-	-	-	++	++	-
<i>L. infantum</i>	-	-	-	-	-	-	-	-	++	++	-
" <i>donorani</i>	-	-	-	-	-	-	-	-	++	++	-
<i>Ctenocephalus canis</i> .	-	-	-	-	-	-	-	-	-	-	-
<i>H. ctenocephali</i>	-	-	-	-	-	-	-	-	-	-	-
<i>Rana pipiens</i> .	-	-	-	-	-	-	-	-	-	-	-
<i>T. rotatorum</i>	-	-	-	-	-	-	-	-	-	-	-

+++, complete inhibition of hemolysis. ++, 50 per cent hemolysis. +, 75 per cent hemolysis. -, complete hemolysis.

The great variability in the fermentative faculty of the different flagellates is at once apparent. Some fermented practically all of the carbohydrates tested, others only a few, and still others none at all. *H. oncopelti* attacked 13 of the 17 carbohydrates tested, *H. lygæorum* only 3. *H. culicidarum* affected 13 carbohydrates, *H. muscidarum* 14, and each had a distinguishing feature, the former fermenting amygdalin but not lactose, the latter slightly attacking lactose but not affecting amygdalin. All the leishmania strains fermented glucose, levulose, mannose, saccharose, and raffinose, and *L. tropica* affected inulin slightly. *H. parva*, the strain from *Calliphora* No. 2, fermented the same sugars as the leishmanias, and galactose in addition. *H. media*, from *Calliphora* No. 1, also resembled the leishmanias but differed from them, and from *H. parva*, in fermenting inulin energetically. It affected galactose only slightly. Only dulcitol and rhamnose were unaffected by any of the flagellates, and of the 18 strains of flagellates tested, *H. ctenocephali* and *T. rotatorium* were the only ones which did not ferment any of the carbohydrates. *H. muscidarum* was the only flagellate to ferment lactose, and arabinose was affected only by *H. muscidarum* and *H. oncopelti*. Amygdalin was fermented only by *H. culicidarum*.

Whether the observed differences will persist during prolonged cultivation remains to be seen.

DISCUSSION.

The classification adopted for the species of *Herpetomonas* isolated in the course of the present investigation requires some comment, in view of the fact that heretofore the custom has been to regard the flagellates found in a given host as specific for that host and to name each strain in a way that indicates its source. If this custom had been rigidly followed, however, it would have been necessary to select several names for organisms which in cultural characteristics, in immunological properties, and in biochemical activity behave in the same way, as, for example, the strains isolated from *Oncopeltus fasciatus*, the Peruvian species of *Oncopeltus*, *Lygæus kalmii* No. 1, *Asclepias syriaca* No. 213, and *Asclepias nivea*. We already had evidence that the strain in *A. nivea* was the same as that in *O. fasciatus*, since the healthy *nivea* plants had been experimentally infected by the feeding

TABLE III.

Fermentation Tests.

Results read after 18 days at 20°C.

Source of flagellate.	Carbohydrate in the medium.										
	Glucose.	Levulose.	Mannose.	Saccharose.	Raffinose.	Inulin.	Galactose.	Maltose.	Saltin.	Xylose.	Mannitol.
<i>O. fasciatus</i>	++	++	++	++	++	++	++	++	++	++	++
<i>Onchopeltus</i> sp. ?.....	++	++	++	++	++	++	++	++	++	++	++
<i>L. kalnii</i> No. 1.....	++	++	++	++	++	++	++	++	++	++	++
" " 2.....	++	++	++	++	++	++	++	++	++	++	++
<i>A. quadrinaculatus</i> ...	++	++	++	++	++	++	++	++	++	++	++
<i>C. pipiens</i>	++	++	++	++	++	++	++	++	++	++	++
<i>M. domestica</i>	++	++	++	++	++	++	++	++	++	++	++
<i>Calliphora</i> sp. ? No. 1,	++	++	++	++	++	++	++	++	++	++	++
" " " 2.	++	++	++	++	++	++	++	++	++	++	++
<i>A. syriaca</i> No. 46.....	++	++	++	++	++	++	++	++	++	++	++
" " " 213....	++	++	++	++	++	++	++	++	++	++	++
" <i>nivea</i>	++	++	++	++	++	++	++	++	++	++	++
Oriental sore.	++	++	++	++	++	++	++	++	++	++	++
<i>L. tropica</i>	++	++	++	++	++	++	++	++	++	++	++
Espundia.	++	++	++	++	++	++	++	++	++	++	++
<i>L. brasiliensis</i>	++	++	++	++	++	++	++	++	++	++	++
Kala-azar.	++	++	++	++	++	++	++	++	++	++	++
<i>L. infantum</i>	++	++	++	++	++	++	++	++	++	++	++
" <i>donovani</i>	++	++	++	++	++	++	++	++	++	++	++
<i>Ctenocephalus canis</i> .	—	—	—	—	—	—	—	—	—	—	—
<i>H. ctenocephali</i>	—	—	—	—	—	—	—	—	—	—	—
<i>Rana pipiens</i> .	—	—	—	—	—	—	—	—	—	—	—
<i>T. rotatorum</i>	—	—	—	—	—	—	—	—	—	—	—

+++++, deep red, turbid (coagulation of serum); ++++, deep red, serum clear; ++, pink, serum clear; +, no change in original bluish color.

seen in the insect gut is usually cylindrical in appearance and is filled with volutin granules; the flagellum is as long as the body or longer, and rather flexible. The flagellates seen in the latex of *A. nivea*, though known to have come from *O. fasciatus*, are charactersitic of the plant type, and the same is true of *H. oncopelti* and *H. lygæorum* as they occur in *A. syriaca*.

In the case of the flagellates isolated from *Calliphora*, we were confronted with the alternative of identifying two strains, distinct morphologically, serologically, and in biochemical activity, with *H. calliphoræ*, or of choosing new names for them. The former course was manifestly unreasonable. The names *H. media* and *H. parva* were selected for these organisms because of the morphological differences between them, but there are differences as well in biological characters whereby the organisms may be identified at any time. We chose a new name also for the strain isolated from *Musca domestica*, because it appeared hardly legitimate to assign to *Herpetomonas muscæ domesticæ* the characteristics which this particular strain had shown.

The flagellates isolated from mosquitoes came from two different host genera, *Anopheles* and *Culex*, but could not be differentiated morphologically, culturally, or in biochemical properties. The name selected for the organism represented by these two strains was *Herpetomonas culicidarum*, which indicates its occurrence in two genera of the family Culicidæ and denotes also the specific properties by which it may be differentiated from the other flagellates of the series, and perhaps also from other flagellates of mosquitoes.

SUMMARY.

Serological reactions and fermentation tests have been employed in the present investigation as a means of differentiating various strains of herpetomonads from one another as well as from leishmanias. The twelve strains of herpetomonads isolated from insects and plants all proved to be serologically unrelated to any of the leishmanias, and were distinguishable from them by the manner in which they affected various carbohydrates.

Three of the strains of herpetomonads tested had been isolated from milkweeds (*Asclepias syriaca* and *A. nivea*) and four from bugs which feed on the latices of these plants (*Oncopeltus fasciatus*, *Oncopeltus sp.?* from Peru, and *Lygæus kalmii*). When tested for their serological

of specimens of *O. fasciatus* known by feces examination to harbor a *Herpetomonas*.⁸ That this same species of flagellate should be present in the normal plant host of *O. fasciatus* (*Asclepias syriaca*) was to have been expected. Nor is it surprising that the same organism should be isolated from another insect which feeds on *A. syriaca*, namely, *Lygæus kalmii*. The infection of this insect could have taken place through ingestion either of infected plant latex or of infected dejecta of *O. fasciatus* deposited on the plant. The ingestion of feces by the bugs has been observed during the course of the experiments, and the insects also have been seen with proboscis inserted into the abdominal cavity of other bugs which were dead or moribund. All the facts, therefore, justify us in regarding these four strains as the same species. The isolation of the same flagellate from the Peruvian species of *Oncopeltus* is perhaps less readily explainable, though not remarkable.

Similarly the facts support the conclusion that the strains isolated from *Lygæus kalmii* No. 2 and *Asclepias syriaca* No. 46 constitute another species. While these two strains showed a slight group reaction toward the anti-*oncopelti* immune serum, they differed greatly from *H. oncopelti* in their action on carbohydrates, fermenting only three sugars instead of thirteen. They average slightly smaller in size, both under natural conditions and in culture, though the morphological differences alone would probably not enable one to discriminate the species. That *Lygæus kalmii* should harbor two types of *Herpetomonas* is not remarkable, and that its plant host should have become infected with both of them is wholly reasonable.

The striking morphological differences among some of the strains under discussion, as they occur in nature, appear to be due to the environment, since they disappear on cultivation under the same conditions. The typical herpetomonad of the plant latex has a flat, ribbon-like body, usually with one or two twists, the cytoplasm is clear, almost hyaline, and the flagellum short—about half the length of the body—and rather straight. The body of the herpetomonad

⁸ These Haitian milkweeds, as already stated in Part I of this report, were presented by Dr. Francis O. Holmes, of the Boyce Thompson Institute for Plant Research, Yonkers, N. Y., who had infected them by allowing infected specimens of *Oncopeltus fasciatus* to feed upon the seed pods.

and carbohydrate-fermenting properties, however, the seven strains proved to be of two kinds only, one represented by the strain first isolated from *Oncopeltus fasciatus* and hence named *H. oncopelti*, the other by *H. lygæorum*, so named because it was first isolated from *Lygæus kalmii*. Serologically there was a certain degree of group reaction among the flagellates of these two types, but in their action upon carbohydrates they were entirely different, *H. oncopelti* splitting thirteen carbohydrates, *H. lygæorum* only three.

Three strains of herpetomonads isolated from flies proved to be distinct both in serological properties and in their action upon carbohydrates. One, derived from the house fly, and called *H. muscidarum*, was able to ferment most of the carbohydrates tested, including lactose which was not affected by any of the other strains. The other two, isolated from bluebottle flies, behaved much the same as the leishmanias with regard to carbohydrate fermentation, attacking five of the same sugars. One of them fermented galactose in addition, the other both galactose and inulin.

Two strains from mosquitoes (*Anopheles* and *Culex*) behaved identically in serological reactions and also in fermentation tests. They are regarded as one species and have been named *H. culicidarum*. This organism ferments thirteen sugars, including amygdalin which no other organism of the series attacks.

One of the most striking phenomena observed was the entire lack of fermentative faculty on the part of *Herpetomonas ctenocephali* and *Trypanosoma rotatorium*. Neither of these organisms was affected by any of the immune sera prepared with other flagellates.

The serological specificity of *Leishmania tropica*, *L. brasiliensis*, and *L. donovani*, and the close relation between *L. donovani* and *L. infantum* were confirmed in the present study. These organisms could not, however, be differentiated by fermentation tests.

The data presented suggest that the biological characteristics of flagellates of the *Herpetomonas* group may be utilized with advantage for identification of a species which occurs in different environments and for separation of different species when they are found in the same environment. If the leishmania parasites pass the flagellated or herpetomonad stage of their life history in some invertebrate host, it may be possible by tests of the sort described to distinguish them from the non-pathogenic herpetomonads which are so widely distributed among insects and plants.

and was operated at 70 kilovolts and 8 milliamperes. Exposures ranged from 20 to 100 minutes at 36 mm. target distance.

Observations.—The most complete color change was obtained in animals that had been exposed for 50 to 100 minutes. The hair in the shaved but unexposed areas began to reappear in about 2 weeks while that in the exposed regions was not visible until 1 week later. When the latter did come in it was entirely white; and after the lapse of 1 month in all it was as long and thick as the rest of the hair (Fig. 1). The hair in the areas exposed from 70 to 100 minutes grew more rapidly than it did in the spots receiving 50 to 60 minute doses. At the end of 3 months the regions rayed for 50 to 60 minutes were rather sparsely covered with hair while the hair in the spots receiving longer exposures grew back in full vigor. The hair in areas exposed for 20 to 50 minutes did not suffer as complete a color change as when the raying had been for longer periods. Only a few scattered hairs in these areas became whitened, the great mass reappearing in the natural color.

2½ months after one of the animals had received 90 and 100 minute exposures upon the abdomen it was noted that a white spot was appearing on its back in a position directly opposite the spot that had received the 90 minute dose (Fig. 2). Under the binocular this white spot, which was slightly larger than the corresponding one on the abdomen, was seen to be made up of a mixture of white and black hairs. The white hairs were not banded, with the outer portion the original black, as might be expected had the hair continued to grow after the hair follicle had lost its power of producing pigment. A second area on the back corresponding to the spot on the abdomen exposed for 100 minutes appeared at first as a white line which after about 10 days extended to form a complete circle. Evidently the rest of the area of exposure had been shielded by the pelvic bones or some other internal obstruction to the rays. Almost 2 weeks after these dorsal white spots appeared on mice rayed for 90 and 100 minutes similar areas appeared on the mice exposed for 70 and 80 minutes. At the time of writing, 3 months after the beginning of the experiment, none of the animals rayed for shorter intervals have developed white spots on the back.

STUDIES ON X-RAY EFFECTS.

XV. THE PREVENTION OF PIGMENT FORMATION IN THE HAIR FOLLICLES OF COLORED MICE WITH HIGH VOLTAGE X-RAY.

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PLATE 16.

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In the course of a study of x-ray dosage it was noted incidentally that the hair of rabbits became white within an area exposed to x-ray produced at high voltage (1). Since color and not the production of hair was affected it was considered desirable to gather more complete data in the hope that it might eventually throw some light on the biological action of the different wave-lengths of x-ray. The results of some preliminary experiments are recorded below.

Material.—In order that any change in color might be most easily detected black haired mice were chiefly used. The hair of such animals is evenly and deeply pigmented.

Methods.—The abdomens of the mice were shaved. The animals were then immobilized on their backs upon boards by binding them on with adhesive tape. Two holes about $\frac{3}{8}$ inch in diameter and about $\frac{1}{2}$ inch apart had been punched in a shield of rubber and lead, and the rubber cement from adhesive tape applied to a small area about the holes by sticking a piece of tape of proper size over the holes and saturating the cloth with xylol. The cloth could then be peeled off leaving its adhesive adhering to the rubber and lead shield. This perforated shield was now adjusted over the shaved abdomen of the mouse where it adhered because of the adhesive. The edges of the shield were fastened to the board by glass thumb tacks. In exposing the shielded animals to the x-ray both holes were left uncovered for the period desired and then the anterior hole was shielded while the posterior opening was rayed for 10 minutes longer. Thus two exposures of different lengths were made on each animal. Two mice were used for each period of exposure employed.

The X-Ray Outfit.—The x-ray machine used was the one designed by Clark (2),

A similar loss of pigment has recently been reported by Coolidge (4) following exposures to the cathode rays. In his cases the hair appeared to grow more rapidly than in the surrounding unexposed area. In the earlier experiments referred to above (1) it was not unusual to find that the hair in an exposed area grew out as a tuft before that in the surrounding shaved area had reappeared. No such increased rate of growth was observed during the present work. Indeed after some of the shorter exposures the growth of the hair was definitely retarded.

SUMMARY.

1. Hard x-rays prevent the formation of pigment in the hair follicles of mice, with result that hair previously black comes in white.

2. Exposure of the ventral side of a mouse to the x-rays causes the hair on the back to come in white about 40 to 50 days after it has whitened the hairs on the abdomen.

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EXPLANATION OF PLATE 16.

FIG. 1. Ventral view of black haired mouse showing the two circular patches of white hair which appeared after exposure of these areas to high voltage x-rays for 90 and 100 minutes.

FIG. 2. Dorsal view of the same mouse showing two areas on the back corresponding to those on the lower side. The dorsal spot is not as well marked for reasons discussed in the text. The whitened hairs appeared about 1 month later than those shown in Fig. 1.

FIG. 3. The skin of the mouse pictured in Figs. 1 and 2 stretched to show in one photograph both the ventral and dorsal spots.

After the hair had grown again to its original length in the exposed areas one of the white spots was shaved. When the hair came in once more it was as white as before.

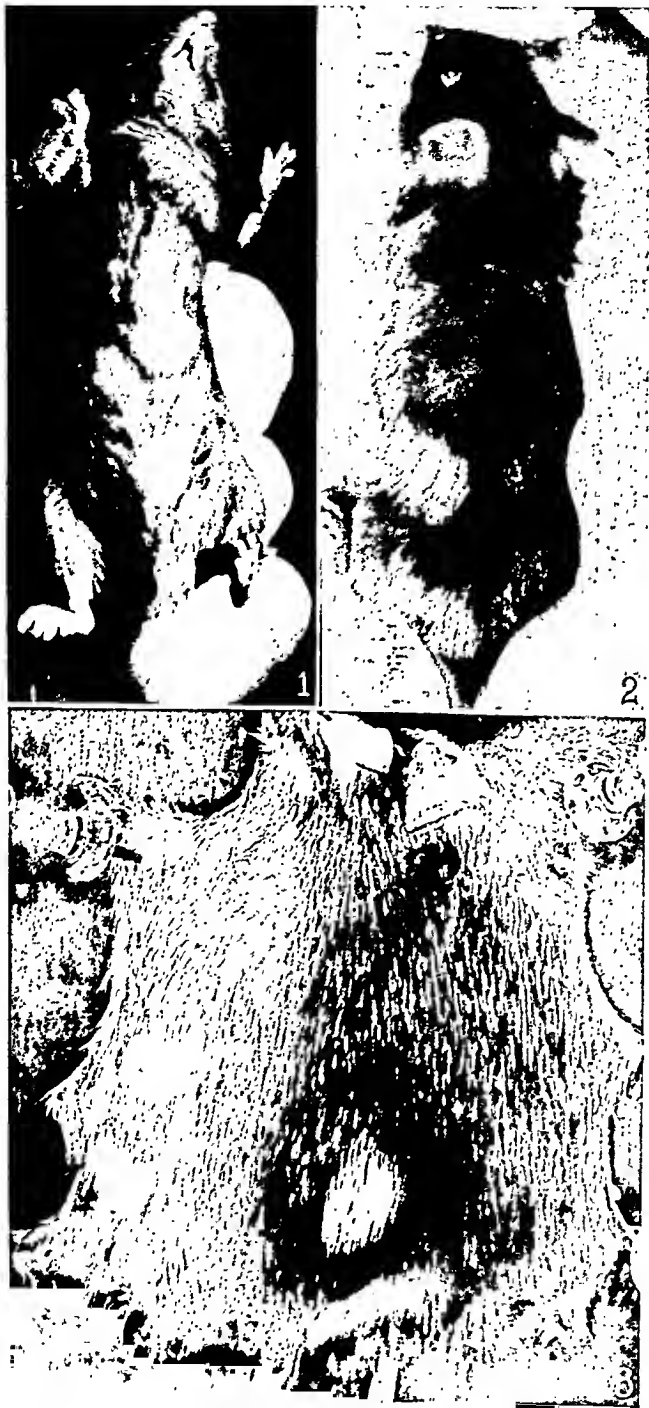
DISCUSSION.

Comparatively little seems to be known about the pigment production in hair. Pigment cells of ameboid shape are found clustered about the papillæ at the base of the follicle and pigment granules have been described within and between the cells of the cortex of the hair of human beings. In addition to the "solid" colors that result from the presence of these granules, pigments in solution are said to stain the cortical cells. Hair is said to be pure white only when pigment is lacking and air is present between the cells of the medulla and the cortex. In cases where air is missing the hair appears grey, never white (3).

Whatever the actual processes responsible for the presence of pigment in hair it is evident that in some way hard x-rays interfere with it permanently. In addition to destroying the color-developing power of the hair, the structure of the cortical cells must be altered in order to permit the entrance of air between them, since otherwise, according to the authority above cited, the hair would not be pure white.

As is evident from the symmetry of the white areas, the effect of the x-rays is due to the direct impingement of them and the injury does not spread beyond the open areas of the shield. Where the effect is seen on the back the force of the rays has been modified by the obstructions to them existing between the ventral and dorsal surfaces. Shorter exposures took longer postexperiment intervals to produce any effects on the pigment in the dorsal hair than did the longer doses.

It is of interest to note the great length of time, $2\frac{1}{2}$ months, between the exposure and the appearance of white hairs on the back of the mouse. There was no dropping out in mass of this hair but the colored hair was gradually replaced with white. This may mean that the changes induced are cumulative. No gradation from black to white was encountered with the ultimate result of an abrupt cessation of pigment production. As far as could be determined none of the hairs were banded, *i.e.* black at the tip and white at the base, but were entirely white.



(Hance and Murphy: Studies on x ray effects. XV.)

the incitant of an epidemic of snuffles and pneumonia in a rabbit stock at Ray Brook, N. Y.¹ We conclude, therefore, that mucoid strains of *Bact. lepidoptericum* have supplanted the "D" strains in the rabbits at the Rockefeller Institute, and are, at present, the prevailing type in at least two other rabbit communities.

Morphology and Staining Characteristics; Colony Formation; Metabolic Activities.

All types of *Bact. lepidoptericum* have the general characteristics of the Pasteurella group. They are small, blunt, Gram-negative, non-motile rods, with deep staining polar bodies. Varying amounts of acid, without gas, are formed within 24 to 48 hours by cultures in peptone broth, containing 1 per cent dextrose, levulose, xylose, saccharose, and mannitol. About 50 per cent of all strains ferment glycerol after 4 to 5 days of incubation. Maltose, lactose, salicin, dulcitol, inulin, arabinose, and rhamnose are not fermented.

Nitrates are reduced to nitrites; the indole test (Ehrlich) is positive. Litmus milk is not acidified.

"D" strains differ somewhat in morphology and staining characteristics from "G" variants and also from mucoid forms and their variants. The special characteristics of each type are found by examining fresh films of living bacteria with dark-field illumination, or by staining them with Wright's modification of Romanowsky's blood stain. Under these conditions, Type "D" cells appear to consist of a rod-shaped, refractile, pink-staining substance, containing two dark, purple-staining polar bodies. They have a slow Brownian movement. Type "G" variants are much smaller: the two polar bodies seem to touch each other, and the refractile, pink-staining, "cytoplasmic" substance is scarcely visible. Brownian movement is rapid. The mucoid forms are twice as large as the "D" types. Their polar bodies are widely separated by the refractile "cytoplasmic" substance which seems to overflow into an outer band or capsule.² Brownian oscillations are slow.

¹ This spontaneous epidemic has been studied by Dr. D. T. Smith, and will be described later in detail.

² This material was not stained with Hiss or Muir stains, nor did Wright's fluid color the "capsules" of pneumococci. Opinions differ as to the presence or absence of "capsules" on bacteria of the Pasteurella group (4).

BIOLOGY OF BACTERIUM LEPISEPTICUM.

III. PHYSICAL, CULTURAL, AND GROWTH CHARACTERISTICS OF DIFFUSE AND MUCOID TYPES AND THEIR VARIANTS.

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We have been engaged in the study of *Bact. lepi-septicum* infection in rabbits with the purpose of finding out the mechanism which governs its natural prevalence. Thus far we have determined three factors which play essential parts: (1) host susceptibility; (2) microbic distribution; and (3) microbic virulence. The first two of these have been discussed in previous papers (1); in this and the following report we shall present the results of our investigations of the infecting agent, *Bact. lepi-septicum*.

Occurrence.

During the past 4 years we have obtained, from rabbits at the Rockefeller Institute animal house, two distinct types of *Bact. lepi-septicum*—so called “D” forms and mucoid forms. De Kruif had previously, in 1920 to 1922, obtained strains of typical rabbit septicemia bacteria from the Rockefeller Institute stock which he observed to dissociate *in vitro* from the original so called “D” type to a “G” type (2); and he later recovered these “G” variants directly from the nasal passages of clinically normal rabbits (3). In 1922 and 1923, we isolated similar “D” and “G” strains from the nasal passages of apparently healthy rabbits, and from rabbits suffering from spontaneous pneumonia, snuffles, and otitis media; but since that time, only mucoid strains and their variants have been recovered. A large percentage of the Rockefeller Institute rabbits has continued to harbor *Bact. lepi-septicum*, but always in the mucoid form. Several hundred cultures, taken from rabbits at a farm in New City, N. Y., over a period of 12 months, were mucoid; and *Bact. lepi-septicum* of the mucoid type proved to be

discoloration beneath the surface of blood agar tube cultures of "D," mucoid, and "G" types, which, according to McLeod, is indirect evidence of the presence of peroxide (9).

TABLE I.

Agglutination of "D," Intermediate, and "G" Cultures of Bact. leipsepticum in Lactate-Lactic Acid Buffer Solution.

Rabbit No. and culture No.	Colony type.	pH range of buffers.									
		4.7	4.5	4.1	3.8	3.5	3.3	3.0	2.7	2.4	Control.
74	D	—	—	—	—	C.	C.	C.	—	—	—
90	"	—	—	—	—	C.	C.	C.	—	—	—
Rivers.	"	—	—	—	—	C.	C.	C.	—	—	—
157	"	—	—	—	—	C.	C.	C.	—	—	—
36	"	—	—	—	—	C.	C.	C.	—	—	—
24	"	—	—	—	C.	C.	++	—	—	—	—
115	"	—	—	—	C.	C.	C.	—	—	—	—
137	"	—	—	—	C.	C.	C.	C.	—	—	—
79	I	—	—	C.	C.	C.	—	—	—	—	—
88	"	—	—	C.	C.	C.	—	—	—	—	—
3 W	"	—	—	C.	C.	C.	—	—	—	—	—
203	"	—	C.	C.	C.	C.	C.	C.	—	—	—
125	"	—	C.	C.	C.	C.	C.	—	—	—	—
179	"	—	C.	C.	C.	C.	C.	C.	—	—	—
186	G	+	C.	C.	C.	+	—	—	—	—	—
201	"	C.	C.	C.	C.	—	—	—	—	—	—
119	"	C.	C.	C.	C.	—	—	—	—	—	—
Rivers.	"	C.	C.	C.	—	—	—	—	—	—	—
188	"	C.	C.	C.	C.	—	—	—	—	—	—
135	"	C.	C.	C.	C.	C.	+	—	—	—	—
35	"	C.	C.	C.	—	—	—	—	—	—	—

— No agglutination.

+ Slight agglutination.

++ Good agglutination; supernatant turbid.

C. Complete agglutination.

Physical Characteristics.

Acid agglutination titrations were made on a large number of freshly isolated cultures of *Bact. leipsepticum*, to compare the isoelectric points of "D" and mucoid types.

Besides these specific differences in morphology, other variations in size occur, which are associated with the age of the culture (5).³ During the period of logarithmic growth of a broth or agar culture of "D," mucoid, or variant strains, individual cells appear very large. This is due, apparently, to an increase in amount, or swelling of the pink-staining, "cytoplasmic" substance. The polar bodies are enlarged also, and stain a reddish purple. Chains, filamentous forms, and other irregularities are numerous. After 24 hours incubation, when the number of organisms is constant, at about 1 billion per cc., the cells become uniform and less than one-half their former size. And finally, after incubation at 37°C. for more than 3 or 4 days, most of the bacteria are still smaller and stain pale blue.

The "D" and "G" colonies have been described by De Kruif as follows: The "D" colonies "were whitish, with rather opaque, glistening centers, fading into translucent outer zones. Their borders were regular. They exhibited marked fluorescence, both by daylight and by artificial light. The surface colonies of Type "G" were somewhat smaller, translucent, and bluish in color, had irregular, serrated edges, and showed little or no fluorescence" (2, 3). In addition to these two colony forms as described, we found, in old broth cultures of "D" strains, a white, opaque colony, slightly fluorescent. From this intermediate colony, a mixture of "D" and "G" types of bacilli was invariably obtained.

Davis (6) has described the colony form of hemorrhagic septicemia bacteria recovered from spontaneous abscesses of rabbits as being moist, viscid, and slightly spreading. The mucoid strains which we have encountered are probably similar. The colonies are large and fluorescent, with whitish, opaque centers and translucent, flowing mucoid margins. The "G" variants of these mucoid strains have a characteristic pin-head colony, convex, bluish, with regular edges, firm in texture, somewhat adherent to the agar, and difficult to emulsify.

Peroxidase is present in less amounts in the "D" and mucoid types than in the "G" variants (7). Peroxide formation could not be demonstrated by direct methods (8); there was, however, greenish

³ Clark has described the same phenomena in other bacterial species (5).

test in which four mucoid cultures were compared with a "D" type, three "G," and one "intermediate" strains. The "D" culture flocculated between pH 3.0 and pH 3.8, the mucoid strains from pH 2.7 to pH 3.8, the "intermediate" from pH 2.7 to pH 4.5, and the "G" from pH 3.5 to pH 4.7.

Type differences in stability of suspension were marked. Mucoid strains did not flocculate completely after 6 to 8 hours centrifugalization at high speed. "D" strains, however, came down in 3 to 4 hours, and "G" strains in $\frac{1}{2}$ to 1 hour.

TABLE III.

Rate of Migration of Bact. leipsepticum, Types "D," "G," and Mucoid in Glycine-Acetate-Phosphate Buffer, pH 7.4.

Type strain.	No. of seconds to travel 83.33 μ .	Potential $\left(\frac{83.33}{\frac{\text{No. sec.}}{5}} \times 14 \right)$.
	<i>sec.</i>	
Rivers "D."	6.3	-37
" "G."	8.9	-26.2
M 555	6.1	-38.2
M 518	5.8	-40.2
Rivers "D"	5.7	-40.9
M 587	6.0	-39.0
M 183	5.7	-40.9

Potential determinations were made with the Northrop type of cataphoresis cell (13). Cell suspensions were washed thoroughly and suspended in glycocoll buffer at pH 7.4, as described by Northrop and De Kruif (11).

The results of these observations are recorded in Table III. Rates of migration of the "D" type and "G" variant were found to be similar to those reported by Northrop and De Kruif; those for the mucoid strains proved similar to the "D" and were considerably higher than the "G" variants.

Growth Requirements and Characteristics.

The different types of *Bact. leipsepticum* fail to grow in buffered asparagine and tryptophane media at atmospheric or reduced oxygen

De Kruif found that the acid agglutination zone of his "D" types was pH 3.0 to 3.5, and of "G" variants, pH 3.5 to 4.7 (10). He noted also that the agglutination zone was affected by the buffer solutions employed.

We employed the standard lactate-lactic acid buffers and technique used by Northrop and De Kruif (11). 16 to 18 hour broth cultures were washed and centrifugalized four times with distilled water. Equal parts of cell suspension and buffer were placed in the water bath at 55° and readings were made at 6 and 18 hours.

TABLE II.
Acid Agglutination of Mucoïd Strains.

Control No.	Colony type.	pH									
		4.7	4.5	4.1	3.8	3.5	3.3	3.0	2.7	2.4	Control.
Rivers.	D	—	—	—	C.	C.	C.	C.	—	—	—
"	G	—	++	C.	C.	C.	++	—	—	—	—
Brown's	"	C.	C.	C.	C.	C.	—	—	—	—	—
"	"	C.	C.	C.	C.	C.	—	—	—	—	—
"	I (intermediate).	—	++	C.	C.	C.	C.	C.	++	—	—
"	M (mucoïd).	—	—	—	++	C.	C.	C.	C.	—	—
DC 30	"	—	—	—	C.	C.	C.	C.	C.	—	—
653	"	—	—	—	C.	C.	C.	C.	++	—	—
544	"	—	—	—	C.	C.	C.	C.	C.	+	—

— No agglutination.

+ Slight agglutination.

++ Good agglutination; supernatant turbid.

C. Complete agglutination.

Table I shows the result of one such test on twenty-one freshly isolated "D," "intermediate," and "G" variant strains. The eight "D" strains flocculated completely between pH 3.0 and pH 3.5; the "G" strains between pH 3.5 and pH 4.7; and six cultures from "intermediate" colonies showed a wide range, characteristic of mixtures of the "D" and "G" types (12).

The acid agglutination zone of mucoïd types proved similar to that of the "D" strains. For example, Table II shows the results of a

ing 5 cc. of plain broth and into others containing the broth plus $\frac{1}{2}$ cc. of a 1/100 solution of hemolyzed rabbit red blood cells. The tubes were then incubated and examined at frequent intervals for evidence of growth.

The results are shown in Table IV. All hemoglobin broth cultures showed maximum growth within 24 hours, indicating that an inoculation of less than ten organisms was sufficient for multiplication. The plain broth cultures of DC 30, DC 12, and DH 5, seeded with 300,000 or more, and No. 544, with 3000 or more, showed good growth at 24 hours. Other plain broth cultures, with an original inoculation of

TABLE IV.

Growth of Mucoid Strains in Plain Broth and Broth Plus Hemoglobin.

Culture No.	Broth medium.	No. per cc. in millions in original cultures.	Dilution.							
			10 ⁻²	10 ⁻³	10 ⁻⁴	10 ⁻⁵	10 ⁻⁶	10 ⁻⁷	10 ⁻⁸	10 ⁻⁹
DC 30	Plain.	360	++	++	48 hrs.	90 hrs.	90 hrs.	90 hrs.	90 hrs.	0
"	Hemoglobin.	360	++	++	++	++	++	++	++	++
DC 12	Plain.	330	++	++	48 hrs.	48 hrs.	48 hrs.	48 hrs.	48 hrs.	
"	Hemoglobin.	330	++	++	++	++	++	++	++	++
DH 5	Plain.	360	++	++	90 hrs.	90 hrs.	90 hrs.	0	0	0
"	Hemoglobin.	360	++	++	++	++	++	++	++	++
544	Plain.	370	++	++	++	++	90 hrs.	90 hrs.	0	0
"	Hemoglobin.	370	++	++	++	++	++	++	++	++

++ = Maximum growth in 24 hours.

less than ten organisms, grew after a lag of from 48 to 90 hours. 9 days later, "G" variants were found in the plain broth cultures of DC 12; the other cultures remained mucoid throughout the 2 weeks period of observation.

This lag in the growth of mucoid strains in plain broth was investigated further by the serial counting method.

Test 2.—A 24 hour plain broth culture of DC 30 was inoculated, in varying amounts, into three 500 cc. Erlenmeyer flasks, each containing 150 cc. of plain broth. Flask 1 received 1 cc. of a 1:100 dilution; Flask 2, 1 cc. of a 1:1000; and Flask 3, 1 cc. of a 1:10,000 dilution. The number of bacilli per cc. in the flasks was

tension, and with or without hemoglobin, iron salts, or other oxygen-absorbing substances. But a 1 per cent peptone solution, or meat extract or infusion broth is adequate for abundant growth of all types, when inoculations of sufficient size are made. In media of the latter sort, specific differences in the manner of growth of "D," "G" variant, and mucoid forms are apparent. The behavior of the "D" and "G" variant strains has been described before, but for the sake of comparison, the main facts may be summarized again as follows:

De Kruif noted that "D" strains, inoculated into broth or peptone solutions, gave rise to "G" variant forms after 48 to 72 hours incubation (2). Later, we made a more detailed study of the growth of this type (14, 15), and noted the following facts: (1) that "D" strains, inoculated into aerobic meat infusion broth, pH 7.4, in quantities less than 100,000 per cc., did not live; (2) that larger seedings multiplied after a period of lag, until a maximum number of 1,000,000,000 per cc. was reached; (3) that after 48 to 72 hours incubation, "G" variants appeared in increasing numbers, until they replaced entirely the original "D" forms. Furthermore, we noted that (4), if the oxygen tension of the broth was lowered mechanically or by the addition of hemoglobin or other iron-containing compounds, inoculations as small as two or three bacilli per cc. invariably multiplied logarithmically, with no appreciable lag until, after 9 to 10 hours, a maximum number of 1,000,000,000 per cc. was reached. Under these latter conditions, the appearance of Type "G" variants was inhibited indefinitely. Finally, we noted that "G" variant strains grew well in broth at either atmospheric or reduced oxygen pressure.

The mucoid strains have now been studied similarly, with the following results.

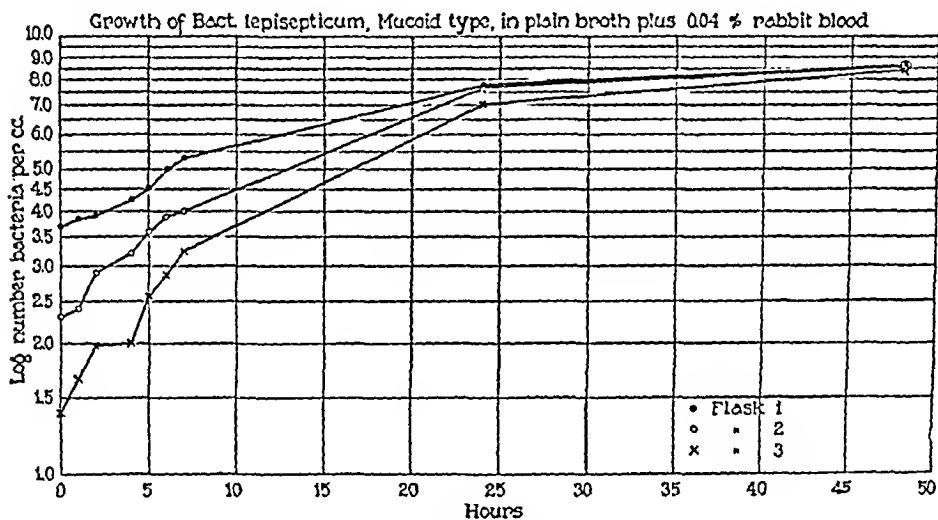
The first test was planned to determine the smallest number of mucoid organisms which would multiply in tubes containing 5 cc. of plain broth, or plain broth plus hemoglobin.

Test 1.—Three mucoid cultures from a rabbit farm at New City, N. Y., and one from the Rockefeller Institute stock were employed. DC 30 and DH 5 were obtained from the nasal passages of animals with snuffles; DC 12 from a lung abscess, and 544 from a healthy nasal carrier. 24 hour plain broth cultures were seeded in $\frac{1}{2}$ cc. amounts, in dilutions varying exponentially from 1 to 9, into tubes contain-

and 24 in Flask 3—increased immediately and at a more or less regular rate for 48 hours, when the maximum counts of 360, 450, and 282 millions per cc. were obtained.

One further experiment was planned to test the ability of sodium pentacyano-aquo-ferroate to act as a substitute for hemoglobin in broth media (13).

Test 4.—Two mucoid strains, DC 30 and DC 12, were grown 17 hours in plain broth and inoculated into 5 cc. tubes of plain broth and plain broth plus 2 per cent sodium pentacyano-aquo-ferroate, in dilutions increasing exponentially from 1 to 9. The two series of tubes were then incubated and examined subsequently for evidence of growth.



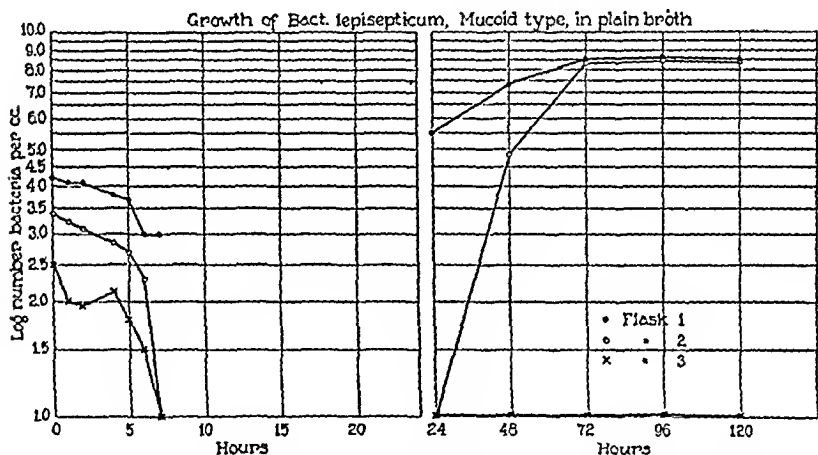
TEXT-FIG. 2. Growth of *Bact. lepi-septicum*, mucoid type, in plain broth plus 0.04 per cent rabbit blood.

Table V shows the result. The mucoid strains grew as readily in the aquo salt broth as in blood broth, while in the plain broth tubes a characteristic lag occurred.

These experiments have emphasized several striking characteristics of the growth of mucoid strains of *Bact. lepi-septicum*. First, a few hundred organisms, or more, inoculated into meat extract broth, survive and, after a lag continuing sometimes for more than 24 hours, multiply at a relatively slow rate, until a maximum number of 300 million to 400 million per cc. is reached. Second, "G" variants of the

determined at once by the plating method; the flasks were then incubated and counts were made at frequent intervals.

The growth curves are plotted in Text-fig. 1. The original number of 15,000 per cc. in Flask 1 dropped, after 7 hours, to 1000; rose at 24 hours to 300,000; and reached a maximum of 300,000,000 per cc. within 72 hours. In Flask 2 the original count of 2460 dropped to zero within 7 hours. The next day, the count was still zero, but on the 3rd day the maximum number of 350,000,000 per cc. was reached. The number of bacteria in Flask 3 dropped within 7 hours from 300 to zero, and no subsequent growth occurred during the 120 hour period of observation.

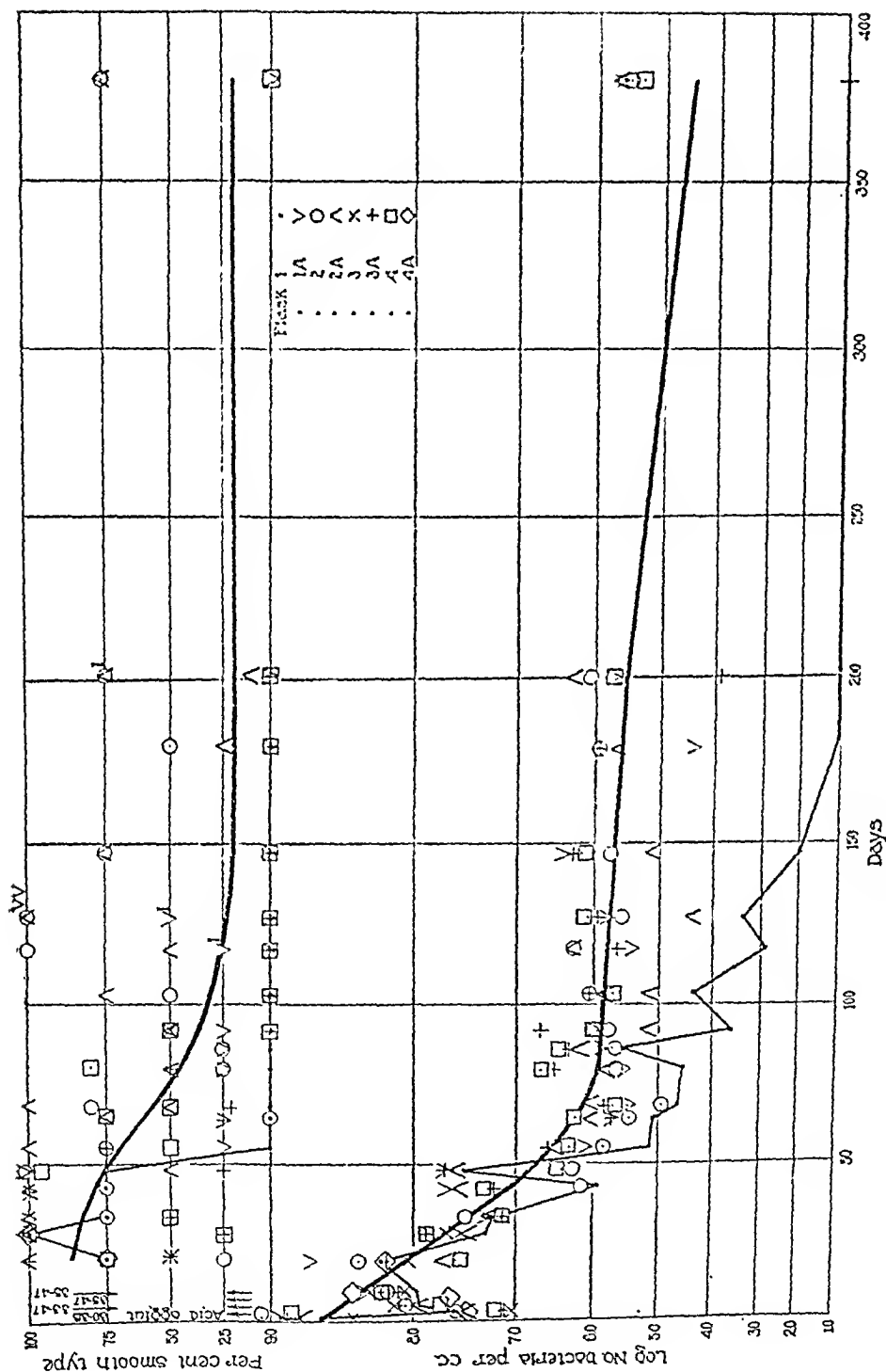


TEXT-FIG. 1. Growth of *Bact. leipsepticum*, mucoid type, in plain broth.

The behavior of the mucoid strain in blood broth was then tested in the same manner.

Test 3.—The same strain, DC 30, was grown for 18 hours in a tube of plain broth and then seeded, in 1 cc. amounts, at dilutions of 1:100, 1:1000, and 1:10,000, into three flasks of 0.04 per cent hemolyzed rabbit blood in 150 cc. of broth. The flasks were incubated and the number of organisms per cc. was determined at frequent intervals by the serial counting method.

The growth curves are shown in Text-fig. 2. The numbers of bacteria inoculated into each flask—4800 in Flask 1; 200 in Flask 2;

TEXT-FIG. 3. Survival of *Bact. lepi-septicum*, "D" type, in plain and blood broth.

mucoïd strains appear slowly, or not at all, under these conditions. Third, hemoglobin or oxygen-absorbing substances added to meat extract broth supply conditions necessary for immediate multiplication after inoculation of less than ten organisms per cc. for 24 to 48 hours, until a maximum number of 300,000,000 to 400,000,000 is reached.

The next experiments were carried out to determine the behavior of "D" and mucoïd cultures over long periods of time.

TABLE V.

Growth of Mucoïd Strains in Plain Broth and Broth Plus Sodium Pentacyano-Aquo-Ferroate.

Culture No.	Medium.	Dilution.									Dissociation.
		10 ⁻³	10 ⁻⁷	10 ⁻⁶	10 ⁻⁵	10 ⁻⁴	10 ⁻³	10 ⁻²	10 ⁻¹	10 ⁻⁰	
DC 30	Plain broth.	++	++	++	4	4					0
"	Plain broth + aquo salt.	++	++	++	++	++	++	++	++		0
DC 12	Plain broth.	++	++	++	+	4	4	4	4	4	+ 7 days.
"	Plain broth + aquo salt.	++	++	++	++	++	++	++	++	++	0

Test 5.—A 15 hour broth culture of the Rivers "D" strain was inoculated in $\frac{1}{2}$ cc. amounts (about 500,000,000 bacteria) into each of eight Erlenmeyer flasks, containing 700 cc. of broth, plus differing amounts of rabbit red blood cells. Flasks 1 and 1 A contained 0.001 as many rabbit red blood cells per cc. as are in 1 cc. whole blood; Flasks 2 and 2 A contained 0.0001 of this quantity; Flasks 3 and 3 A, 0.00001 as much; and Flasks 4 and 4 A no blood. These cultures were then incubated for 12 months. From time to time, counts were made, acid agglutination titrations were carried out, type dissociation percentages were estimated, and atypical colonies and other phenomena studied.

The results are summarized in Text-fig. 3, and may be stated approximately as follows: 2 days after inoculation, the acid agglutina-

of the cell and serum mixtures was reduced to pH 6.5, that it became necessary to employ the "G" variant of each strain for the actual titrations. De Kruif found that "G" variants were antigenically similar to their homologous "D" strains (2), and we have observed this relationship between mucoid strains and their "G" variants. The variant "G" strains flocculated completely in serum dilutions of 10^3 or more, whereas the parent "D" or mucoid strains agglutinated with difficulty in serum dilutions as low as 10^2 .

TABLE VI.

Direct and Cross-Agglutination of "G" Variants of "D" and Mucoid Strains of Bact. lepi-septicum.

Antisera.	Culture.							
	Rivers "G"	No. 103 "G"	No. 329 Mucoid "G"	No. 544 Mucoid "G"	No. DC 12 Mucoid "G"	No. DC 30 Mucoid "G"	Snuffles (1) Mucoid "G"	Snuffles (4) Mucoid "G"
Rivers "D".....	3840*	960	0	0	0	0	0	0
No. 103 "D".....	960	960	0	60	0	0	0	0
" 329 mucoid.....	0	0	120	1920	120	480	960	480
" 544 "	0	0	0	480	960	240	480	960
" DC 12 mucoid.	0	240	470	3840	960	240	960	3840
" DC 30 "	0	0	1920	480	960	3840	60	—
Snuffles (1).....	0	0	480	3840	—	—	120	60
" (2).....	0	0	480	480	—	—	60	240
" (3).....	0	0	120	0	—	—	120	60
" (4).....	0	0	1920	960	—	—	240	1920

* Dilution limit of distinct (+) agglutination.

— No titration.

Two "D" cultures, Rivers and No. 103, and four mucoid strains, Nos. 329, 544, DC 12, and DC 30, were compared. In addition to these, mucoid strains from the nasal passages of four stock rabbits affected with chronic snuffles were used, as well as serum from each of these animals. Direct and cross-agglutinations were made wherever possible and repeated a number of times. A control titration of the homologous strain and serum was included in each series of determinations.

The results of these several tests are summarized in Table VI. The two "D" antisera agglutinated the "G" variants of the "D" but none of the "G" variants of the mucoid strains to any significant

tion range of each culture was still in the "D" zone; on the 3rd and subsequent days it had widened to include both the "D" and "G" zones. This change was interpreted as indicating the appearance of "G" variants in the cultures. Further estimates made by plating methods showed a gradual decrease in the numbers of "D" colonies, and an increase in the "G" colonies. On the 200th day two of the remaining six flasks still contained "D" colonies. This and the slow rate of bacterial dissociation in plain broth are to be explained by the relatively large volume of broth and low oxygen tension in the flask used in this experiment. "Intermediate" colonies were noted in large numbers after the 100th day.

On the 34th day Flasks 3 and 4 A became contaminated and were discarded. The bacterial counts in five of the six remaining flasks were in close agreement, decreasing regularly from about 200,000,000 per cc. on the 5th day, to 3,000,000 on the 70th day. On the 200th and 377th days the counts averaged about the same. The count in Flask 1, however, dropped steadily after the 50th day and reached zero on the 180th day. This discrepancy cannot be explained at present.

Under the conditions of this experiment, therefore, the "D" type of *Bact. lepi-septicum* survived for many months. During this period, the number of viable organisms per cc. dropped from its maximum to a point of approximately constant level, and at the same time, "intermediate" and "G" variants replaced the original "D" forms.

The curve of survival of mucoid cultures over long periods of time was similar to that of the "D" cultures; "G" variants appeared more slowly, however.

Serological Studies.

The antigenic properties of "D" and mucoid types were compared by means of direct and cross-agglutination tests.

Sera were prepared as follows: 17 hour blood broth cultures, heat-killed and living, washed once in saline, were injected intravenously or subcutaneously into rabbits which had not been previously exposed to *Bact. lepi-septicum*. The animals were bled when the titre of the serum reached a maximum.

Agglutination tests were made with bacterial suspensions, washed four times in distilled water. The homologous strains flocculated so poorly, even when the pH

to the amount of original seeding. This is considerably longer than that observed for the "D" strains. The period of multiplication is relatively long—24 to 48 hours—and the maximum number per cc. does not exceed 600,000,000. "D" strains reach 1,000,000,000 per cc. within 24 hours. "G" variants appear very slowly in mucoid cultures as contrasted with "D" strains, where their occurrence is rapid and abundant. The presence in the broth of blood or other oxygen-absorbing substances enables less than ten mucoid organisms per cc. to multiply without lag and to reach a maximum number of about 600,000,000 per cc. in 24 to 48 hours. "D" strains reach a count of 1,000,000,000 per cc. within 12 hours under similar conditions.

9. "D" and mucoid strains, inoculated into 750 cc. of plain or blood broth, and incubated at 37°, remain alive for more than 12 months. The numbers per cc. decrease to about 1/1000 of the maximum after the first 6 weeks. Subsequently, the rate of decrease is slower. The original strains are gradually replaced by "intermediate" and "G" colonies.

10. The various mucoid strains proved similar in their antigenic properties. "D" strains were also mutually related, but no cross-agglutination between "D" and mucoid types was demonstrable.

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amount, while the four mucoid sera flocculated the six variant mucoid cultures, but practically none of the "D" variant strains. The four sera from the snuffles rabbits agglutinated the mucoid variant but not the "D" variant strains. The "D" variant cultures behaved similarly in the various sera; the mucoid variant cultures were also quite regular in this respect, except for No. 544 and snuffles (1), which appeared to flocculate more readily than the others.

These tests and similar ones on many other cultures show that "D" cultures differ from mucoid cultures in their antigenic properties but are themselves mutually related.⁴

A further consideration of the data presented will be given in the next paper of this series.

SUMMARY.

1. From 1922 to 1926, mucoid types of *Bact. leprosepticum* have proved more prevalent than "D" types in certain rabbit communities.

2. The bacteria from the mucoid colonies are larger than the "D" type; "G" variants are very small. All types are largest during the period of logarithmic growth. Differences in size appear to be determined chiefly by the amount of "cytoplasmic" substance.

3. The mucoid colony is larger and appears watery as compared with the "D" colony.

4. Fermentation tests, indole and nitrate reduction reactions of mucoid and "D" types and their variants are similar.

5. The acid agglutination zone of the mucoid type is similar to that of the "D" type.

6. The mucoid type is more stable in suspension than the "D" type.

7. The negative potential of mucoid types is similar to that of the "D" and higher than that of "G" variants.

8. The behavior of the mucoid type of bacilli in fluid media differs from that of the "D" strains in the following respects. About 500 or more mucoid organisms per cc., inoculated into plain broth, will grow; "D" strains require seedings of at least 100,000. A lag of 3 to 48 hours duration occurs in the growth of mucoid strains, according

⁴ Recent publications by A. Tanaka, *J. Infect. Dis.*, 1926, xxxviii, 389 to 429, state that the antigenic properties of various strains of *Pasteurella* are similar. Apparently, he has experienced no difficulty in obtaining agglutination of his various strains; our experience and that of De Kruif have been quite the contrary.

Two possible explanations for the variability of animal reaction as just illustrated must be considered, namely, that it is due either to chance or to actual differences in the resistance of each individual. Topley (2) and Lockhart (3), on the basis of statistical analyses,—which we believe to be incorrectly applied—assume that the potential reaction of each animal is the same and is constant, variations in their behavior being due entirely to chance. We, on the other hand, are convinced as result of the pathological and bacteriological evidence obtained through the observation of experimental mouse typhoid (1, *b*, *c*) and rabbit septicemia infections (1, *d*) that animals react differently to bacteria because of differences in their resistance, that those which die soon after injection do so because they are relatively susceptible, and that those which escape infection do so because they are especially resistant. Consequently, until direct evidence to the contrary is forthcoming, we shall regard the reaction of the host to the presence of injurious influences as a standard of measurement which varies in each animal according to its resistance.

As one comes to recognize that the amount of resistance differs among individuals (1, *b*, *c*, *d*), races (4), and at seasons of the year (5), that it is affected by diet (1, *e*), light, and other environmental conditions, and consequently that in a virulence titration this host reaction, which we take as the standard of measurement, reflects differences in animal susceptibility as well as bacterial potentialities, one comes also to appreciate the necessity of rigid measures of control during experimentation. As many variables as possible must be eliminated. An inbred race of animals (1) should be used, whose (2) environmental conditions have been uniform and such that they have not been exposed (3) to the specific organism. As large numbers of them as possible (4) of similar age and weight should be employed for each titration. Dosage should be measured in terms of numbers of organisms given to each animal. And finally, if the true, or native, pathogenicity of a microbe under natural conditions is to be determined, the native host should be employed and the bacteria administered by way of the normal portal of entry.

Unfortunately, experimental methods are governed to no small extent by working facilities. Thus, while our own virulence determinations of mouse typhoid bacilli have been carried out under con-

BIOLOGY OF BACTERIUM LEPISEPTICUM.

IV. VIRULENCE OF DIFFUSE AND MUROID TYPES AND THEIR VARIANTS.

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In the preceding paper (1, a) we dealt with a number of physical, chemical, and biological characteristics of *Bact. leipsepticum*; in this present one we shall take up the question of virulence,—its relation to certain other bacterial characteristics and to the occurrence of the natural infection.

Virulence of "D" and Muroid Types and Their Variants.

At the present time, there is but one method of estimating the virulence of a microorganism,—that of animal inoculation. The technique is to administer the specific microbe to a number of laboratory animals, and to observe their subsequent behavior over a fixed period of time. The effects produced are usually recorded in terms of the duration of life of the animals inoculated, but under some conditions, in those of the number of animals that become infected or in types and severity of infection. The investigator's interpretation of such results depends upon his attitude toward certain complicating factors. Chief among these is the difference in reaction of the individual animals to the microorganism. If a group of animals is injected at one time with the same dose of pathogenic bacteria, certain ones may die, others survive with signs of chronic infection, and still others prove refractory. Those that die may succumb after different intervals of time, and those that survive show varying types and degrees of infection. Thus it is evident that the method of titrating virulence by means of animal inoculation is essentially an attempt to evaluate an unknown in terms of an arbitrary standard, which is itself ill defined and subject to wide variation.

had died from spontaneous septicemia. The "G" variant was obtained by cultivating the "D" strain 72 hours in plain broth (1, g). The mucoid strains were freshly isolated in 1925 from snuffles and nasal carrier rabbits (1, d).

Eleven titrations of the Rivers "D" strain have been made over a period of more than 2 years. In nine instances the mortality from

TABLE I.

Virulence of "D," Mucoid, and "G" Strains of Bact. leipsepticum Instilled Intranasally into Rabbits.

Strain.	Experiment.	Date.	Total No. used in experiment.	Pneumonia and septicemia.		Snuffles.		Carriers.		Resistant.	
				No.	Per cent.	No.	Per cent.	No.	Per cent.	No.	Per cent.
Rivers "D".....	1	1923	6	2	33.2	4	66.4	0	—	0	—
".....	2	1923	6	1	16.6	5	83.4	0	—	0	—
".....	3	1923	6	2	33.2	2	33.2	1	16.6	1	16.6
".....	4	1924	6	3	50	0	—	1	16.6	2	33.4
".....	5	1924	12	3	25	8	66.7	1	8.3	0	—
".....	6	1924	12	8	66.7	2	16.6	0	—	2	16.6
".....	7	1924	7	1	14.3	1	14.3	4	57.2	1	14.3
".....	8	1925	11	5	27	6	54	1	9	1	9
".....	9	1925	19	2	10	11	55	2	10	4	20
".....	10	1925	5	1	20	2	40	2	40	0	—
".....	11	1925	8	2	25	2	25	1	12.5	3	37.5
Total average, Rivers "D".....			98	28	28.5	43	44	13	13.3	14	14
Rivers "G".....	1	1925	11	0	—	0	—	0	—	11	100
Mucoid 544.....	1	1925	10	0	—	2	20	6	60	2	20
" 329.....	1	1925	10	0	—	4	40	5	50	1	10
" DC 30.....	1	1925	8	0	—	3	37.5	3	37.5	2	25
Total average.....			28	0	—	9	32.3	14	50	5	17.5

pneumonia and septicemia was between 10 and 33 per cent; in two, over 50 per cent. The average mortality was 28.5 per cent; chronic snuffles developed in an average of 44 per cent, and 13.3 per cent became chronic nasal carriers of the organisms. Two other "D" strains

ditions such as have been just outlined (1, b), the titrations of rabbit septicemia organisms described in the present paper have been limited by the small numbers of rabbits available and by the fact that they were not strain-inbred. We have used animals in groups of 8 to 10, of similar age and weight, bred at the Rockefeller Institute, not previously exposed to *Bact. lepi-septicum*; and we have instilled intranasally a known number of organisms. This procedure has been followed by results which have been relatively constant and which indicate, we believe, the true or natural virulence of different strains of *Bact. lepi-septicum*. Furthermore, the results have served as controls in estimating the reliability of other titration methods, less accurate, but more practical. Thus it was found that when a culture given intranasally to rabbits caused a high percentage of pneumonia and septicemia cases, it likewise proved fatal when injected in small numbers intrapleurally into rabbits, or intraperitoneally into mice, and conversely that strains showing little effect when given to rabbits by the normal portal of entry were less apt to kill when introduced parenterally. Hence these latter procedures were used to gain presumptive evidence of differences in the pathogenicity of a large number of strains of *Bact. lepi-septicum* over varying periods of time.

Intranasal Inoculation of Rabbits.

In 1921, De Kruif (6) reported that "D" strains of *Bact. lepi-septicum* are more virulent than their "G" variants, that the virulence of the "D" type is relatively constant, but that Type "G" virulence may be somewhat increased by rabbit passage. Since that time, we have compared the virulence of many "D" and mucoid strains and their variants, isolated from nasal carrier rabbits and from rabbits with abscesses, snuffles, otitis, pneumonia, and septicemia.

The results of a number of what we shall call "natural" virulence titrations of "D," mucoid, and "G" variant strains are summarized in Table I.

In each instance, 250,000,000 organisms of a 17 hour blood broth culture from the stock agar slant were instilled into the right and left nares of rabbits of similar age—5 months—and weight—800 to 1000 gm.—from the Rockefeller Institute breeding room. The Rivers "D" strain was isolated in 1923 from a rabbit which

TABLE III.

Virulence of "D" and "G" Types of *Bact. leipsepticum* Injected Intraperitoneally into Mice.

Strain.	Date.	Average No. of bacteria injected.							
		10 ³	10 ⁷	10 ²	10 ⁵	10 ⁴	10 ³	10 ²	10 ¹
Rivers "D"	1/14/24	1*			2-2		2-2		3-3
"	1/ 7/24							2-S.	
"	1/ 9/24	1-1					1-2-S-S.	1-S.	4-S-S-S.
"	1/15/24		1-		1-1-1-1-	1-1	1-1-1-	1-1-2-	1-1
	1/25/24				1-1-1-1-	1-1	1-1-1-	3-3-3-	
					1-S.	1-1-1-	1-1-1-	3-3-3-	
						3-3-4-	2-2-2-	3-4-4-	
						S-S-S.	2-3-4-	4-5-S-	
							S-S.	S-S-S.	
	2/ 5/24	1-1				2-3		1	
"	3/ 5/24			1-1		1-1		2-2	
"	4/ 5/24			1-1		S-S.		S-S.	S-S.
"	12/12/24				1-				1-2
"	1/ 3/25		1-1						
"	1/13/25			1-1			1-S.		
"	1/30/25			1-1			S-S.		
"	2/ 7/25		1-1						
"	2/17/25				1-3				
"	3/ 4/25				1				
"	3/10/25				S-S.				
"	4/ 7/25				S-S.				
"	4/17/25				S-S.				
"	5/ 7/25				1-1				
"					1-1				

from cases of spontaneous pneumonia showed a similar and apparently constant degree of virulence.¹

The titration of the Rivers "G" strain included in Table I indicates the almost total lack of what we term "natural" virulence of this variant. None of the 11 rabbits used in the test became infected.

Typical intranasal titrations of three mucoid strains are also shown in Table I. None of the animals died; 20 to 40 per cent, average 32.3 per cent, developed chronic snuffles, and 37.5 to 60 per cent, average 50 per cent, became chronic carriers of the bacteria.

These results show the relative degree of virulence of "D" and mucoid types of *Bact. leproseptica* and their variants under natural conditions of infection, indicating besides that the "D" type tends

TABLE II.

Virulence of "D," Mucoid, and "G" Types of Bact. leproseptica Injected Intrapleurally into 800 to 1000 Gm. Rabbits.

Type.	No. of organisms injected.				
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
"D".....	†2 days.	3	5	8	12±*
Mucoid.....	6	6	8	10±*	
"G".....	6	6	10±*	10±*	

† Duration of life in days after injection.

* About 50 per cent of the animals survived. An average value indicating duration of life was difficult to obtain.

either to kill its host or to induce chronic snuffles, that the mucoid type is relatively non-lethal, but induces snuffles and carrier cases, while the "G" variant is practically without effect. Furthermore, they furnish evidence that the virulence of different strains of the same type is relatively speaking the same and that it is constant under natural conditions.

Intrapleural Inoculation of Rabbits.

Rabbits similar in age and weight to those of the preceding series were employed. The cultures were taken from stock blood agar

¹ Two illustrative titrations of Strain 103, Type "D," are described in full in the *J. Exp. Med.*, 1926, xliii, 573.

slants, grown overnight in blood broth, and injected into the right chest cavity. Bacterial counts were made by the dilution method.

In Table II are summarized the effects of twelve titrations of "D" strains on 96 rabbits, six titrations of "G" strains on 30 rabbits, and six titrations of mucoid strains on 48 rabbits. Although less striking, the results are similar to those obtained from the nasal instillation of the cultures. "D" strains killed in the shortest period of time, mucoid strains were less effective, while "G" strains proved least virulent. Different strains of the same type behaved similarly; no profound fluctuation in virulence of any type-pure strain was observed.

Intraperitoneal Inoculation of Mice.

We have pointed out that a number of titrations on freshly isolated strains and strains used over long periods of time were made by the method involving the intraperitoneal injection of mice. A 17 hour blood broth culture was suitably diluted, counted, and then injected intraperitoneally into mice 12 to 14 weeks old, from the Rockefeller Institute breeding room. Whenever these freshly isolated strains were tested, the Rivers "D" stock culture was titrated simultaneously as a control.

Table III summarizes the titrations of "D" and "G" strains. The Rivers "D" strain has been tested twenty-six times in this manner over a period of 2 years. No significant fluctuations in virulence are apparent. 1 million organisms or more killed mice regularly within 24 hours; 100,000 in 1.4 days on the average, with 24 per cent survivors; smaller doses acted accordingly. Two other "D" cultures showed similar high virulence. The Rivers "G" variant and four other "G" strains failed to kill when less than 10,000,000 organisms were given. No change in the virulence of these strains was observed.

58 titrations of 36 mucoid strains are grouped chronologically in Table IV. The results of the titrations of the Rivers "D" strain, carried out simultaneously with each series of mucoid strains as a control, are omitted from this table and grouped in Table III. They may be identified by comparing the respective dates of each experiment.

The eight mucoid cultures designated by capital letters came from snuffles, carrier, and pneumonia rabbits at a farm in New City, N. Y. The *leptisepticum*

"	6/30/25	1-1	1-1	3-3	8-S. S-S. S-S. 4-S.	1-S. 2-S.	2.6 days: 30% S.	2.1 days: 41% S.
"	8/20/25	1-1	1-1					
"	11/18/25	1-1	1-2					
"	11/19/25	1-1	1-2					
"	11/20/25	1-1	2-2					
"	12/ 9/25	1-1	1-2					
"	12/15/25	1-1	1-2					
Average....		1 day.	1.2 days.	1 day.	2 days: 37% S.	1.6 days: 37% S.	2.6 days: 30% S.	2.1 days: 41% S.
203 "D"	2/ 5/24	1-1						
115 "D"	2/ 5/24	1						
Rivers "G"	1/ 7/24	1-1	S-S. 2		2-S. 2-2 S-S.		2-1 2-3 S-S.	
"	1/10/24	1-1	1-S.	S-S.				
"	1/15/24	1	1-S.	S-S.				
Average....		1	1.5-60% S.	S.	S.		S.	
88 "G"	2/ 5/24	1-S.						
157 "G"	2/ 5/24	1-1						
188 "G"	2/ 5/24	1-1						
79 "G"	2/ 5/24	1-1						
186 "G"	2/ 5/24	1-S.						

* Numerals indicate duration of life in days after injection.

S. = Survived 30 days.

TABLE IV—*Concluded.*

Strain.	No. of organisms injected.						
	Date.	10 ⁷	10 ⁸	10 ⁹	10 ¹⁰	10 ¹¹	10 ¹²
485 (2)	11/19/25	1-1	S.-S.		S.-S.		
485 (3)	12/ 9/25	1-2	2-S.			2-3	
399 (1)	6/30/25			1-1			S.-S.
399 (2)	11/20/25	1-1		3-4			3-4
399 (3)	12/ 9/25	1-2		S.-S.			2-2
587 (1)	8/20/25	1-1			8-S.		
587 (2)	11/19/25	1-1	4-4		2-4		
555 (1)	11/19/25	2-3		S.-S.		S.-S.	
555 (2)	12/ 9/25	1-S.		S.-S.			3-S.
492	11/18/25	1-S.	S.-S.		S.-S.		
369	11/18/25	1-2	2-S.		S.-S.		
556	11/18/25	S.-S.	S.-S.		S.-S.		
349	11/18/25	1-1	2-3		S.-S.		
395 (1)	11/20/25		1-1		S.-S.		
395 (2)	12/ 9/25	1-1-2-S.	S.-S.- S.-S.	S.-S.		2-S.	3-S.
338	11/20/25	2-2		S.-S.			
516	12/15/25	1-2-2- 2-2-4		2-S.-S.- S.-S.-S.			S.-S.-S.- S.-S.-S.
Total average.....		1.4 days: 14.5% S.	2 days: 60% S.	1.7 days: 75% S.	4 days: 80% S.	2.9 days: 69% S.	2.8 days: 75% S.

S. = Survived 30 days.

Numerals = Duration of life in days after injection.

infection is endemic there among a stock of about 2500 rabbits. Strains designated as "Smith 1" and "72" came from cases of pneumonia in a spontaneous epidemic among a group of rabbits at Ray Brook, N. Y., and were kindly supplied by Dr. D. T. Smith. Strains numbered in the three, four, and five hundreds came from a special group of rabbits at the Rockefeller Institute. The source and treatment of these rabbits were as follows. 60 animals from the Institute breeding room, not previously exposed to *Bact. lepi-septicum* infection, were placed in separate cages in a small, special room, in December, 1924. Caretakers and attendants were allowed free access, so that infection from the other Rockefeller Institute stock rabbits might be introduced. Nasal swab cultures were taken at frequent intervals and the types of organisms present were studied. Some of the virulence titrations on the mucoid strains recovered in this way have been included in this and the following tables.

TABLE IV.

Virulence of Mucoïd Strains of Bact. leprosepticum Injected Intraperitoneally into Mice.

Strain.	No. of organisms injected.						
	Date.	10 ²	10 ⁴	10 ⁵	10 ⁶	10 ⁷	10 ⁸
BBB 24	12/17/24			S.-S.			3-S.
DC 30	12/17/24			S.-S.			5-S.
DC 66	12/17/24			3-S.			S.-S.
590	1/ 3/25		S.-S.			S.-S.	
652	1/ 3/25			S.-S.			S.-S.
560 (1)	1/ 3/25		S.-S.			S.-S.	
560 (2)	2/ 7/25		S.-S.			1-3	
560 (3)	4/17/25		1-2			S.-S.	
545	1/13/25		S.-S.			S.-S.	
569 (1)	1/13/25		S.-S.			4-5	
569 (2)	2/ 7/25		1			3-3	
569 (3)	6/30/25			1-1		S.-S.	
544 (1)	1/13/25		S.-S.			S.-S.	
544 (2)	1/30/25		S.-S.			S.-S.	
544 (3)	2/17/25		S.-S.			S.-S.	
544 (4)	3/10/25		S.-S.			S.-S.	
544 Rab (5)	3/10/25		S.-S.			S.-S.	
329 (1)	2/ 7/25		1-1			1-3	
329 (2)	3/10/25		4-S.			3-4	
329 Rab (3)	3/10/25		1-1			4-4	
329 (4)	11/20/25		3-4		S.-S.		S.-S.
321 (1)	2/17/25		2-S.			1	
321 (2)	11/18/25	1-1		2-S.		4-S.	
375	2/17/25	1-1				3-S.	
DC 47	3/ 4/25		2-2			S.-S.	
DC 335	3/ 4/25	1-1			S.-S.		
DC 672	3/ 4/25		S.-S.			S.-S.	
BC 7	3/ 4/25		S.-S.			S.-S.	
DC 72	3/ 4/25		4-S.			2-S.	
536	4/ 7/25		S.-S.			S.-S.	
543	4/ 7/25		S.-S.			S.-S.	
Smith (1)	4/ 7/25		1-S.			S.-S.	
541 (1)	4/17/25			S.-S.			S.-S.
541 (2)	11/19/25	1-1	3-S.		S.-S.		
81	4/17/25		S.-S.			S.-S.	
Smith (72)	5/ 7/25		1-S.			S.-S.	
577	6/30/25		1-1				S.-S.
557 (1)	6/30/25		1-1				S.-S.
557 (2)	11/19/25	1-1	4-4		2-4		
557 (3)	12/ 9/25	S.-S.		S.-S.			S.-S.
485 (1)	6/30/25		1-1				S.-S.

TABLE V.

"Intraperitoneal Titrations" of Mucoid Strains in Mice.

Culture.	Dilution.	No. of organisms injected.	Duration of life in days for the 3 mice.	Average duration of life.	Average No. of survivors.
3/23/26 Rivers.	10^{-1}	104,000,000	0.7-0.7-0.7	0.7	
"	10^{-2}	10,400,000	0.7-0.7-2	1.1	
	10^{-3}	1,040,000	0.7-0.7-2	1.1	
	10^{-4}	10,400	2-7-S.	4.5	1
563	10^{-1}	60,800,000	0.7-0.7-4	1.8	
	10^{-2}	6,080,000	2-2-S.	2.0	1
	10^{-3}	608,000	4-S.-S.	4.0	2
	10^{-4}	6,080	3-6-S.	4.5	1
518	10^{-1}	105,000,000	0.7-0.7-5	2.1	
	10^{-2}	10,500,000	0.7-2-2	1.6	
	10^{-3}	1,050,000	2-2-5	3.0	
	10^{-4}	10,500	2-2-4	2.6	
330	10^{-1}	130,800,000	0.7-0.7-2	1.1	
	10^{-2}	13,080,000	0.7-2-S.	1.3	1
	10^{-3}	1,308,000	2-S.-S.	2.0	2
	10^{-4}	13,080	S.-S.-S.		3
329	10^{-1}	68,400,000	0.7-0.7-2	1.1	
	10^{-2}	6,840,000	4-6-S.	5.0	1
	10^{-3}	684,000	5-6-S.	5.5	1
	10^{-4}	6,840	2-5-S.	3.5	1
379	10^{-1}	87,000,000	0.7-2-3	1.9	
	10^{-2}	8,700,000	2-8-S.	5.0	1
	10^{-3}	870,000	1-4-11	5.0	
	10^{-4}	8,700	8-10-13	10.0	
3/24/26 Rivers.	10^{-1}	105,000,000	0.7-0.7-1	0.8	
	10^{-2}	10,500,000	0.7-1-6	2.6	
	10^{-3}	1,050,000	2-6	4.0	
	10^{-4}	10,500	0.7-6-S.	6.5	1
565	10^{-1}	72,000,000	1-3-4	2.6	
	10^{-2}	7,200,000	0.7-2-4	2.2	
	10^{-3}	720,000	0.7-S.-S.	0.7	2
	10^{-4}	7,200	1-2-3	2.0	

The data in Table IV bring out one fact clearly, namely, that the mucoid type of *Bact. leprosepticum* is consistently less virulent for mice than the "D" and slightly more virulent than the "G" type. 1 million mucoid organisms killed an average of 40 per cent of the animals in 2 days and failed entirely to kill 60 per cent. Smaller doses were without effect in more than 68 per cent of the animals. Furthermore, the table indicates that mucoid strains, living in the nasal tissues of rabbits, do not change in pathogenicity; cultures presumably of the same mucoid strains obtained on two, three, or four different occasions from Rabbits 560, 569, 544, 329, 321, 541, 557, 485, 399, and 587 proved to be similar in virulence. Although no significant differences in the virulence of these strains were apparent, it seemed wise to test this question further by the following method.

A series of "intraperitoneal titrations" upon mice was carried out with twenty-seven mucoid strains recovered from the nasal passages of rabbits in the special room at the Rockefeller Institute, referred to above. Cultures obtained from rabbits within 4 days of the time of the experiment were given intraperitoneally to 12 mice, in the following dilutions: 10^{-1} , 10^{-2} , 10^{-3} , and 10^{-5} . The Rivers "D" strain, the virulence of which is known, was administered each day to a similar group as a control. The number of organisms given to each mouse was determined by the plating method. Duration of life was noted and the healthy survivors discarded after 14 days.

The results of these tests are recorded in Table V, and are rearranged in Table VI to show the average duration of life of mice receiving similar doses of different strains. In spite of every precaution, irregularities in survival time occurred. Some cultures which seemed to show a relatively great killing power in one dilution were less active in other dilutions. Frequently, 1 of the 3 mice given a certain dose of culture survived over a period of time out of all proportion to that of its mates. These irregularities could not have been due to microbic factors, and it is hardly possible that chance factors alone would lead to such discrepancies. We believe the inconsistencies to be due to differences in the resistance of the mice, and accordingly that the significance of the results must be determined by inspection alone.

Clearly, the Rivers "D" strain is more virulent than the mucoid cultures; it killed the mice in the shortest period of time in various doses. Furthermore, Cultures 518, 560, 555, 512, and 369, which

TABLE V—Continued.

Culture.	Dilution.	No. of organisms injected.	Duration of life in days for the 3 mice.	Average duration of life.	Average No. of survivors.
557	10 ⁻³	768,000	S.-S.-S.		3
	10 ⁻⁵	7,680	S.-S.-S.		3
383	10 ⁻¹	78,000,000	4-5-S.	4.5	1
	10 ⁻²	7,800,000	S.-S.-S.		3
	10 ⁻³	780,000	4-S.-S.	4.0	2
	10 ⁻⁵	7,800	6-9-S.	7.5	1
593	10 ⁻¹	147,000,000	2-2-2	2.0	
	10 ⁻²	14,700,000	4-4-S.	4.0	1
	10 ⁻³	1,470,000	3-3-5	3.6	
	10 ⁻⁵	14,700	5-7-S.	6.0	1
375	10 ⁻¹	120,000,000	0.7-0.7-2	1.1	
	10 ⁻²	12,000,000	2-4-S.	3.0	1
	10 ⁻³	1,200,000	0.7-3-3	2.2	
	10 ⁻⁵	12,000	6-7-S.	6.5	1
368	10 ⁻¹	103,800,000	0.7-3-3	2.2	
	10 ⁻²	10,380,000	3-6-S.	4.5	1
	10 ⁻³	1,038,000	4-S.-S.	4.0	2
	10 ⁻⁵	10,380	9-9-S.	9.0	1
346	10 ⁻¹	75,000,000	4-4-S.	4.0	1
	10 ⁻²	7,500,000	3-S.-S.	3.0	2
	10 ⁻³	750,000	5-S.-S.	5.0	2
	10 ⁻⁵	7,500	S.-S.-S.		3
3/26 Rivers.	10 ⁻¹	156,000,000	0.7-0.7-0.7	0.7	
	10 ⁻²	15,600,000	0.7-2-2	1.6	
	10 ⁻³	1,560,000	2-2-2	2.0	
	10 ⁻⁵	15,600	2-3-S.	2.5	1
535	10 ⁻¹	70,400,000	0.7-2-2	1.2	
	10 ⁻²	7,040,000	2-3-4	3.0	
	10 ⁻³	704,000	2-S.-S.	2.0	2
	10 ⁻⁵	7,040	8-S.-S.	8.0	2
492	10 ⁻¹	162,000,000	2-5-6	4.3	
	10 ⁻²	16,200,000	2-5-7	4.6	
	10 ⁻³	1,620,000	S.-S.-S.		3
	10 ⁻⁵	16,200	2-S.-S.	2.0	2

TABLE V—Continued.

Culture.	Dilution.	No. of organisms injected.	Duration of life in days for the 3 mice.	Average duration of life.	Average No. of survivors.
555	10 ⁻¹	68,400,000	0.7-0.7-3	1.6	
	10 ⁻²	6,840,000	1-2-3	2.0	
	10 ⁻³	684,000	0.7-2-S.	0.9	1
	10 ⁻⁴	6,840	3-S-S.	3.0	2
462	10 ⁻¹	120,000,000	0.7-0.7-3	1.5	
	10 ⁻²	12,000,000	0.7-1-4	1.9	
	10 ⁻³	1,200,000	2-3-S.	2.5	1
	10 ⁻⁴	12,000	2-2-S.	2.0	1
395	10 ⁻¹	84,000,000	2-4-S.	3.0	1
	10 ⁻²	8,400,000	2-5-S.	3.5	1
	10 ⁻³	840,000	5-6-S.	5.5	1
	10 ⁻⁴	8,400	6-13-S.	9.5	1
544	10 ⁻¹	42,000,000	0.7-1-4	1.9	
	10 ⁻²	4,200,000	0.7-7-2	1.1	
	10 ⁻³	420,000	3-S-S.	3.0	2
	10 ⁻⁴	4,200	3-13-S.	9.5	1
543	10 ⁻¹	46,800,000	S-S-S.		3
	10 ⁻²	4,680,000	3-S-S.	3.0	2
	10 ⁻³	468,000	5-S-S.	5.0	2
	10 ⁻⁴	4,680	S-S-S.		3
556 A	10 ⁻¹	117,000,000	0.7-3-4	2.6	
	10 ⁻²	11,700,000	1-3-5	3.0	
	10 ⁻³	1,170,000	1-5-S.	2.0	1
	10 ⁻⁴	11,700	3-3-5	3.6	
3/25 Rivers.	10 ⁻¹	120,000,000	0.7-0.7-0.7	0.7	
	10 ⁻²	12,000,000	2-2-S.	2.0	1
	10 ⁻³	1,200,000	2-2-2	2.0	
	10 ⁻⁴	12,000	2-3-3	2.6	
512	10 ⁻¹	69,000,000	0.7-2-2	1.6	
	10 ⁻²	6,900,000	0.7-2-6	2.9	
	10 ⁻³	690,000	2-3-6	3.6	
	10 ⁻⁴	6,900	2-4-1-S.	3.0	1
557	10 ⁻¹	76,800,000	0.7-2-S.	1.3	1
	10 ⁻²	7,680,000	S-S-S.		3

TABLE VI.
Average Duration of Life of Mice Receiving Similar Doses of Different Mucoid Strains of *Bact. leipsepticum*.

Culture.	10 ³		10 ⁵		10 ⁶		10 ⁸		10 ⁹		10 ¹⁰	
	Duration of life in days.	No. survi-ors.	Duration of life in days.	No. survi-ors.	Duration of life in days.	No. survi-ors.	Duration of life in days.	No. survi-ors.	Duration of life in days.	No. survi-ors.	Duration of life in days.	No. survi-ors.
Rivers.	0.7, 0.8, 0.7, 0.7: = 0.7 av.	1	1.1, 2.6, 2, 1.6 = 1.4 av.	1	1.1, 4, 2, 2, = 2.3 av.				2.6, 2.5, 4.5, 6.5 = 4.0 av.	3 = 0.7 av.		
518	2.1		1.6		3.0				2.6		2.3	2
560			1.6		3.0		3.3				3.0	
555			1.6		2.0		0.9	1			3.0	1
512			1.6		2.9		3.6				6.0	1
369			1.1		3.6		2.3	1			9.5	1
544			1.9		1.1		3.0	2				
462	1.5		1.9		2.5	1			2.0	1		
535			1.2		3.0		2.0	2			8.0	2
563			1.8		2.0	1	4.0	2			4.5	1
329			1.1		5.0	1	5.5	1			3.5	1
379			1.9		5.0	1	5.0				10.0	
565			2.6		2.2		0.7	2			2.0	
183			2.0		3.3		2.6				6.3	
556	2.6		3.0	1	2.0	1			3.6			
375	1.1		3.0		2.2				6.5	1		
593	2.0		4.0	1	3.6				6.0	1		
395			3.0	1	3.5	1	5.5	1			9.5	1
368			4.0	1	3.3		7.0	1			3.0	
330	1.1		1.3	1	2.0	2			—	3		

TABLE V—*Concluded.*

Culture.	Dilution.	No. of organisms injected.	Duration of life in days for the 3 mice.	Average duration of life.	Average No. of survivors.
560	10^{-1}	51,600,000	0.7-2-2	1.6	
	10^{-2}	5,160,000	2-3-4	3.0	
	10^{-3}	516,000	2-3-5	3.3	
	10^{-6}	5,160	2-2-3	2.3	
369	10^{-1}	40,000,000	0.7-0.7-2	1.1	
	10^{-2}	4,000,000	2-5-7	3.6	
	10^{-3}	400,000	2-5-S.	2.3	1
	10^{-6}	4,000	5-7-S.	6.0	1
587	10^{-1}	42,000,000	S.-S.-S.		3
	10^{-2}	4,200,000	S.-S.-S.		3
	10^{-3}	420,000	S.-S.-S.		3
	10^{-6}	4,200	2-S.-S.	2.0	2
349	10^{-1}	39,000,000	3-3-3	3.0	
	10^{-2}	3,900,000	2-4-S.	3.0	1
	10^{-3}	390,000	S.-S.-S.	3-S.	3
	10^{-6}	3,900	2-S.-S.	2.0	2
368	10^{-1}	51,600,000	4-4-S.	4.0	1
	10^{-2}	5,160,000	2-3-5	3.3	
	10^{-3}	516,000	4-10-S.	7.0	1
	10^{-6}	5,160	3-3-S.	3.0	1
183	10^{-1}	40,000,000	2-2-2	2.0	
	10^{-2}	4,000,000	2-3-5	3.3	
	10^{-3}	400,000	2-3-3	2.6	
	10^{-6}	4,000	3-5-11	6.3	

S. = Surviving and clinically healthy at 14 days.

proved lethal within 1 to 3 days when given in doses of 10^7 to 10^8 , and to some of the mice in doses as small as 10^3 , would seem from this experiment to be more pathogenic than Cultures 587, 557, 492, 346, and 349, which, in doses of 10^7 to 10^8 killed usually after 3 days and in smaller doses were generally without effect. Nevertheless, we wished to gain further evidence of these differences by more direct methods. We therefore chose an apparently virulent mucoid strain, No. 518, another less so, No. 369, and a third of little virulence, No. 383 as

determined by the intraperitoneal method, and instilled a 24 hour blood broth culture of each into the nares of ten 600 to 800 gm. rabbits from the Rockefeller Institute breeding room. About 125,000,000 organisms were introduced into each naris. The results of this test are shown in Table VII.

3 weeks after infecting the animals, the carrier rate and percentage of resistant animals in each group were similar. 2 animals in the 369 series, however, died from pleuropneumonia. This relationship still obtained after 6 and 9 weeks; hence we concluded that these strains showed about the same degree of natural virulence for rabbits, and that they were similar to the other cultures studied (Table I).

TABLE VII.

Virulence of Mucoïd Strains of Bact. leipsepticum Instilled Intranasally into Rabbits.

Date,	Group.	Specific mortality.	Snuffles.	Carriers.	Refractory animals.
		<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
4/4/26 infected.					
4/24/26	383	0	11.1	67	33
"	369	20	0.0	75	25
"	518	0	12.5	62	38

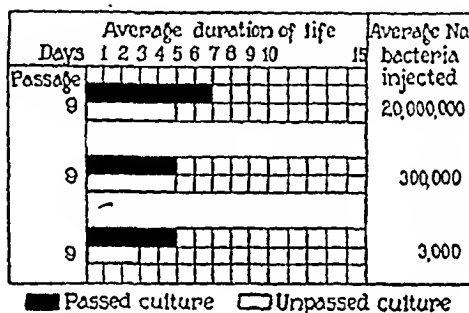
Effect of Artificial Cultivation and of Animal Passage on "D," Mucoïd, and "G" Types.

In the experiments so far described, we have noted several times that the virulence of "D," mucoïd, or "G" types does not fluctuate significantly. That the virulent "D" organism gives off less virulent "G" variants was observed *in vitro* by De Kruif (6) and by us in experiments involving infection of the nasal passages of rabbits (1, d). But nothing has been noted up to the present that would indicate the occurrence of the reverse process, namely, the change from a less virulent to a more virulent type. We undertook to follow any type changes which might occur, first, by growing the bacteria in various artificial media, and second, by making animal passages.

Artificial Cultivation.—Since the change from the virulent "D" or mucoïd type to the avirulent "G" variant is brought about when the oxygen tension of the media is high, and inhibited when it is low (1,

exudate was also instilled into the nares of each of 4 rabbits. At the same time, a 6 hour rabbit blood broth culture from the original, unpassed stock culture was injected intrapleurally into 4 rabbits in 1 cc. quantities, undiluted, 1:10, 1:100, and 1:1000; and 4 rabbits were given the undiluted culture intranasally in doses of $\frac{1}{2}$ cc. The actual number of bacteria per cc. in each culture was determined by the plating method.

None of the rabbits inoculated intranasally showed clinical signs of infection. Those injected intrapleurally, however, died at intervals of 5 to 7 days (Text-fig. 1). And the average duration of life of animals receiving the "passed" culture was at least as long as those injected with the "unpassed" culture. It is concluded, therefore, that under the conditions of this experiment, 9 pleural passages did not affect the intrapleural or intranasal virulence of the "D" strain, R. 63, of *Bact. leipsepticum*.



TEXT-FIG. 1. Virulence of 9 passage culture and "unpassed" stock culture of *Bact. leipsepticum*. "D" strain, R. 63.

"Average number bacteria injected" represents the mean of the nearest comparable figures.

Experiment 2.—Another virulent "D" strain, Rivers, previously described, was grown 17 hours in rabbit blood broth. 1 cc. was then injected intrapleurally into a 1000 gm. rabbit. This animal died the following day. A heart's blood broth culture from this rabbit, incubated 14 hours, was injected intrapleurally into 6 rabbits in the following dilutions: 1:1, 1:10, 1:1000, and 1:100,000. The number of bacteria per cc. was estimated by the usual plating method. 6 additional rabbits were injected intranasally with the undiluted culture.

2 animals receiving the nasal injections died with pleuropneumonia; 4 of the 6 developed snuffles; 2 remained well. Of those receiving the culture intrapleurally 1, with the 600,000,000 dose, died in 1 day; 1, with 60,000,000, in 1 day; 2, with 600,000, in 2 days; and 2, with 6000, in 1 and 2 days.

1 cc. of the pleural fluid of the first rabbit to die was injected intrapleurally into a 1000 gm. rabbit, and the number of bacteria thus injected was determined as before. A similar direct pleural passage was continued through 7 rabbits with the

g), it seemed desirable to ascertain whether "G" or mucoid types may change to more virulent forms when grown in media of low oxygen tension. Hence, a number of strains was seeded into plain broth and into broth enriched with yeast extract, vegetable, or animal tissue, in which the oxygen tension was reduced mechanically, or by the addition of blood, potato, iron salts, and oxides (2). Rapid and slow passages were tried at different temperatures, but the results were entirely negative. Neither type specificity nor grade of virulence was altered.

Animal Passage.—Experiments 1 to 5 were planned to test the effect, if any, of direct passages through the rabbit, without artificial cultivation, of types of *Bact. lepi-septicum* introduced into the body in an abnormal way.

TABLE VIII.

Direct Intrapleural Rabbit Passage of Bact. lepi-septicum, "D" Type, R. "63."

Rabbit No.	No. of passages.	Culture injected.	Quantity injected.	Duration of life in days after injection.
			cc.	
1	1	18 hr. blood broth.	1	2
1 A		18 " " "	1	5
2	2	Pleural exudate: Rabbit 1.	1	7
3	3	" " " 2.	1	5
4	4	" " " 3.	1	5
5	5	" " " 4.	1	1
6	6	" " " 5.	1	1
7	7	" " " 6.	$\frac{1}{2}$	2
8	8	" " " 7.	$\frac{1}{2}$	3

Experiment 1.—A culture of *Bact. lepi-septicum*, Type "D," R. 63, obtained from the nasal passages of a rabbit at the onset of spontaneous snuffles, was grown for 18 hours in rabbit blood broth, and injected in 1 cc. amounts into the left pleural cavity of two 800 to 1000 gm. rabbits. 2 days later 1 rabbit was dead; on the 5th day the second rabbit died. 1 cc. of the pleural exudate of the first rabbit was injected directly into the left pleural cavity of a third rabbit of the same size. 7 days later this rabbit died, and 1 cc. of its pleural exudate was injected as before into a fourth 800 gm. rabbit. This procedure was continued for 8 direct rabbit passages. The results, summarized in Table VIII, indicate that the duration of life decreased somewhat with the intrapleural passage of the culture.

The pleural exudate was then taken from Rabbit "8" and injected directly, without artificial cultivation, intrapleurally into 4 rabbits in the following broth dilutions: 1:1, 1:100, 1:10,000, and 1:1,000,000. $\frac{1}{2}$ cc. of the undiluted pleural

Of the 6 rabbits receiving the passed culture intranasally, 1 died with pleuropneumonia and the other 5 developed snuffles. Of the 6 rabbits receiving the unpassed culture intranasally, 2 died with pleuropneumonia and 2 more developed snuffles.

The results of the intrapleural inoculation are summarized in Text-fig. 2. The chart includes the results of the preliminary control titration, as well as those of the passed and unpassed cultures, and shows that there was no significant difference in duration of life between animals receiving the "passed" and those given the "unpassed" cultures. Hence, it is concluded that animal passage, as described in this experiment, does not affect the virulence of the "D" Rivers strain.

Experiment 3.—A culture of *Bact. lepi-septicum*, Type "G," was obtained from the nares of a rabbit at the onset of spontaneous snuffles, and designated as Strain "R. 82." It was grown for 16 hours in blood broth and then injected intrapleurally

TABLE X.

Direct Pleural Passage of Bact. lepi-septicum, "G" Strain, "R. 82."

Rabbit No.	Material injected.	No. of bacteria injected.	Duration of life.
1	1 cc. blood broth culture.	470,000,000	7 days.
2	1 " pleural exudate: Rabbit 1.	200,000,000	5 "
3	1 " " " " 2.	5,000,000,000	1 day.
4	$\frac{1}{2}$ " heart's blood: " 3.	?	3 days.
5	1 " pleural exudate: " 4.	?	1 day.
6	1 " " " " 5.	?	12 days.
7	Culture from abscess in chest wall of Rabbit 6.	?	2 "
8	1 cc. pleural exudate: Rabbit 7.	?	6 hrs.
9	1 " bloody pleural fluid: Rabbit 8.	?	4 days.
10	1 " pleural exudate: Rabbit 9.	?	2 "

into six 1000 gm. rabbits in the following dilutions: 1:1, 1:10, 1:1000, and 1:100,000. The actual number of bacteria injected was determined by the plating method. The animal receiving 470,000,000 bacteria lived 7 days; the one receiving 47,000,000 survived; the 2 given 470,000 died in 5 days; and the 2 given 4700 survived.

From the first rabbit of this series to die, 1 cc. of pleural exudate was taken and injected intrapleurally into another rabbit of similar weight. A bacterial count was also made. This procedure was then repeated through a series of 10 rabbits as shown in Table X. Probably over 1 billion bacteria were injected in every case. The survival times varied from 6 hours to 12 days.

From the tenth passage rabbit, 1 cc. of pleural exudate was taken and injected intrapleurally into 6 rabbits in the following dilutions: 1:20, 1:200, and 1:20,000. At the same time, a 16 hour rabbit blood culture from the unpassed strain was injected into 6 similar rabbits in the following dilutions: 1:2, 1:20, and 1:2000.

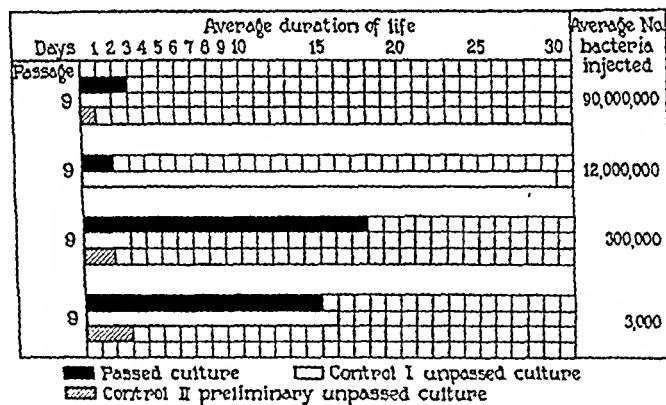
result that most of the rabbits received over a billion organisms and were dead within 24 to 48 hours (Table IX).

The pleural exudate of the rabbit of the eighth passage was then injected intrapleurally into 6 rabbits in the following dilutions: 1:100, 1:1000, 1:100,000,

TABLE IX.

Direct Intrapleural Rabbit Passage of Bact. lepi-septicum, "D" Type, Rivers.

Rabbit No.	Material injected.	No. of bacteria injected.	Duration of life in days.
1	1 cc. blood broth culture.	600,000,000	1
2	1 " pleural exudate: Rabbit 1.	1,700,000,000	3
3	1 " " " " 2.	1,000±	2
4	1 " " " " 3.	9,200,000,000	1
5	1 " " " " 4.	85,000,000,000	1
6	1 " " " " 5.	120,000,000,000	2
7	1 " " " " 6.	30,000,000,000	1
8	1 " " " " 7.	200,000,000	1



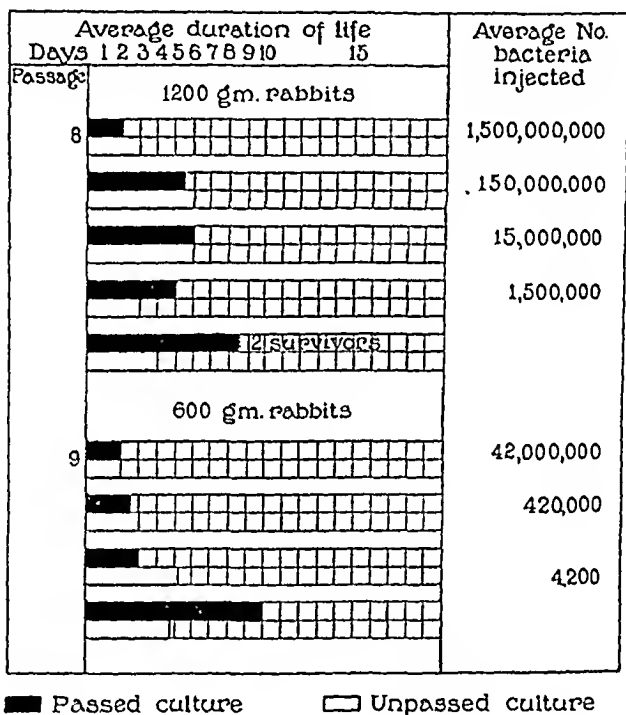
TEXT-FIG. 2. Virulence of 9 passage culture and "unpassed" stock culture of *Bact. lepi-septicum*. Rivers "D" strain.

"Control (II)" represents preliminary titration (Table II).

and 1:10,000,000. 6 rabbits were injected intranasally with the undiluted culture. At the same time, an 18 hour rabbit blood broth culture from the unpassed stock, Rivers strain, was injected intrapleurally as follows: 1:10, and 1:100, 1 rabbit each; 1:10,000, and 1:1,000,000, 2 rabbits each. 6 rabbits were also injected intranasally with this culture. The number of organisms per cc. in the original cultures was determined as usual.

material died on the 1st and 4th days; the next 2 on the 1st and 2nd; the next on the 1st and 2nd, and the seventh passage couple on the 1st and 2nd days. Plates from all these animals showed pure cultures of typical mucoid colonies.

Pleural fluid from the first seventh passage rabbit to die was diluted in salt and given intrapleurally to 10 rabbits. An additional 10 rabbits were injected in a similar manner with the unpassed stock culture. Finally, from 1 of the rabbits receiving this seventh passage culture, pleural fluid was again diluted and given to 8 more animals. 8 controls were given the unpassed culture at the same time. Counts were made and the duration of life of the rabbits receiving the passed cul-



TEXT-FIG. 4. Virulence of 8 and 9 passage culture and "unpassed" stock culture of *Bact. leipsepticum*. Mucoid strain, 329.

ture was compared to that of the animals which had been given a similar number of the unpassed bacteria.

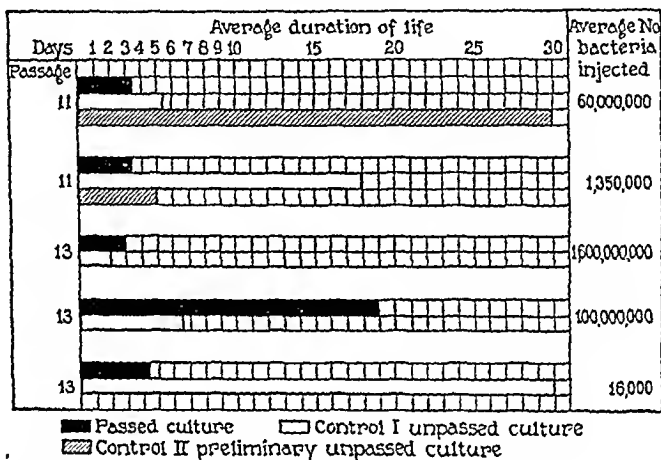
The results of these titrations are plotted in Text-fig. 4, and show that no significant difference occurred in the survival time of animals receiving the "passed" and "unpassed" cultures.

We conclude, therefore, that this method of rapid animal passage does not change the virulence or type characteristics of the mucoid *Bact. leipsepticum*.

A summary of the results, plotted in Text-fig. 3, shows that in the two instances in which the numbers of bacteria injected were at all comparable, the animals receiving the "passed" culture died sooner than those of the "unpassed" series.

This apparent change in virulence was tested further by a second titration. The "passed" culture and pleural fluid from the first rabbit to die in the above titration series were injected directly into the thoracic cavity of another animal. After one more direct passage, the culture from the pleural fluid was inoculated into 6 rabbits in dilutions of 1:10, 1:100, 1:10,000, and 1:1,000,000, and the unpassed control culture was inoculated in dilutions of 1:1, 1:10, 1:1000, and 1:100,000.

The results of this titration are also given in Text-fig. 3, and in this case show that animals given the passed culture lived somewhat longer than the controls



TEXT-FIG. 3. Virulence of 11 and 13 passage culture and "unpassed" stock culture of *Bact. leprosepticum*. "G" strain, R. 82.

given quantitatively similar doses of the unpassed culture. One must conclude, therefore, when the results of the entire experiment are considered, that animal passage did not induce a significant change in the virulence of Culture "G" No. 82.

Experiment 4.—A mucoid strain, No. 329, obtained from a case of spontaneous snuffles, was passed in the same manner. 800 gm. rabbits from the breeding room were used and the material was introduced intrapleurally in $\frac{1}{2}$ cc. amounts. Each rabbit dying of the infection was autopsied with care, and cultured to see if any type changes were occurring.

The first animal received $\frac{1}{2}$ cc. of an 18 hour blood broth culture, and died on the following day. $\frac{1}{2}$ cc. of its pleural exudate was then given to 2 more rabbits. They died on the 1st and 2nd days following. Pleural fluid from the first one to die was given to 2 more; they died on the 2nd day. Those receiving the fourth passage

bred at the Rockefeller Institute under uniform environmental conditions, and free of previous exposure to *Bact. lepidpticum*, together with the technique of intranasal instillation of similar doses,—were sufficient to give consistent and relatively uniform effects. These we have regarded as presumptive evidence of the following: (1) that of the three types of *Bact. lepidpticum* described, “D” strains are the most virulent or pathogenic, mucoid forms less so, and “G” types little or not at all virulent; (2) that different strains of the same type are of similar virulence; and (3) that passage of type-pure strains through non-toxic, nutrient media, or through animals, does not modify their virulence.

The experiments described in the present communications were planned to investigate certain microbic factors which seem to influence the spread of *Bact. lepidpticum* infection. Accordingly, we studied the types encountered in nature, their behavior in the rabbit host, and other qualities which might possibly be related to their parasitic activities.

At the outset we discovered that very little is known about the “Pasteurella” or “hemorrhagic septicemia” organisms. Every small, blunt bacillus with bipolar granules and certain quite definite fermentative reactions is placed in this group and is further designated by prefixing the name of the animal from which it was obtained. Differentiation on the basis of serological studies has been extremely difficult and as yet not sufficiently detailed and complete to be of use. We have, therefore, called our strains “*Bact. lepidpticum*,” because they belong to the Pasteurella group and were obtained from rabbits, but at the same time have recognized the possibility that similar forms may also be present in other animal hosts.

The “D” and mucoid types may or may not be mutually related. Each has its distinctive growth characteristics and antigenic properties, but these differences are not sufficient to exclude the possibility that the two types may have resulted from the action of bacteriophage or so called “mutation” phenomena upon a single “parent” strain.

We have attempted to explain the difference in natural prevalence of “D” and mucoid types in terms of virulence and vegetative capacity (1, *a*, *d*). Thus we find that “D” types are at present rarely encountered in rabbits, while the mucoid forms are widespread. The

The above experiments indicate that passage of *Bact. lepi-septicum* through rabbits, its native host, by way of an abnormal portal of entry, does not affect its virulence. In the following tests, animal passage was effected by way of the normal portal of entry, that is, the nares and upper respiratory tract.

Experiment 5.—Rabbits used in this experiment came from the Rockefeller Institute breeding room, the entire population of which has been free of snuffles and *Bact. lepi-septicum* carriers for 2 years. The "D" culture, "Rivers," was employed.

Six 800 gm. rabbits were given $\frac{1}{2}$ cc. of an 18 hour rabbit blood broth culture into each external naris by means of a blunt syringe. The fluid was introduced without touching the mucous membranes.

3 days later, 1 rabbit died of pleuropneumonia. An 18 hour heart's blood broth culture was given in the same manner to 2 more rabbits. 1 of these showed signs of snuffles after 24 hours; the other died 9 days later of pleuropneumonia. A blood broth culture from this rabbit was given intranasally to 3 more rabbits. 2 of them developed snuffles and 1 died after 9 days with pleuropneumonia. From this animal, a heart's blood broth culture was taken and after 17 hours' incubation was given in similar doses of $\frac{1}{2}$ cc. to 3 more animals. These 3 rabbits remained clinically well. Similar results were obtained with other "D," mucoid, and "G" strains.

It is concluded, therefore, that under the conditions of these experiments, the passage of *Bact. lepi-septicum* through a series of hosts of the native species, by way of the normal portal of entry, does not increase the virulence of the organism.

DISCUSSION AND SUMMARY.

As a preface to a general discussion of these experiments, we wish to refer again briefly to the titrations of virulence described in this paper. None of them was carried out in an altogether proper manner,—it was necessary to employ rabbits of mixed breed, to use small numbers, and in some instances, to compare the results of titrations done at different times of the year. Furthermore, many tests were made in the mouse, a foreign host, and by means of artificial injection into the peritoneal cavity. Hence, if our results had shown fluctuations and wide differences, we should have experienced difficulty in interpreting their significance.

Fortunately, however, the control measures which we were able to employ,—namely the utilization of animals of similar age and weight,

adaptable to a vegetative existence. The virulence of the different strains of each type appears to be about the same and not to be affected by passage through animals or non-toxic, nutrient media. Although each possesses distinctive and constant characteristics, we feel that the possibility of mutual relationship has not been entirely excluded. Investigation of bacterial properties associated with virulence has as yet given no definite and positive results.

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former prove to be highly virulent and slightly vegetative; the latter, less virulent and readily vegetative. The same inverse relationship between virulence and prevalence obtains in human diphtheria (7) and pneumococcus infections (8);—virulent Type I and II pneumococci and diphtheria bacteria are relatively uncommon in carriers, while the less pathogenic diphtheria forms and Type III² and IV pneumococci are widespread. It is possible, therefore, that in these respiratory diseases the virulent types of bacteria are transients, unable to survive in a community except at epidemic times, while the less pathogenic types are more adapted to a parasitic existence in the tissues of a considerable percentage of a population (9).

Just what properties of bacteria are responsible for their ability to kill or to survive indefinitely in the surface tissue of an animal has not been determined. We find that physical properties such as agglutinability in acid buffer solutions, stability in suspension, and potential, bear no consistent relationship to virulence and vegetative capacity. Qualitative fermentation and indole reactions, likewise, gave no significant results. Experiments relating to comparative growth rates and nutritional requirements have, however, shown the following differences: Virulent "D" types were found to require a medium with relatively low oxygen tension and these multiplied with extreme rapidity; mucoid types were less exacting and grew more slowly; while "G" variants needed no accessory substances for good growth. Hence, there may prove to be some relation between food supply, metabolic activity, and pathogenicity; bacteria may be parasitic or saprophytic because of their own specific nutritional requirements, and may be virulent in one host and not in another because of chemical differences in the available food. It will be interesting to test the validity of these suggestions by further experiment.

We may summarize the results of this investigation as follows. A "D" and a "mucoid" type of *Pasteurella* organisms have been recovered from rabbits; the former is relatively rare, is virulent, and not vegetative; the latter is very common, less virulent, and readily

² We consider Types III and IV to be less virulent than Types I and II because of the relatively low ratio of mortality per cent to incidence per cent. We also note a very interesting experimental study by M. J. Rosenau and his associates which tends to confirm this belief (*Am. J. Hyg.*, 1926, vi, 463).

methyl, and isopropyl alcohol, acetone, and acetic acid, all yielded precipitates which showed decided activity. When a comparison was made between these precipitates and the original extract, none was found to be quite as active as the original. However, this was not surprising for, if a sufficient concentration of precipitant such as acetic acid or alcohol was used, some of the protein became denatured as could be seen by a difference in the translucency of its solution. When a sufficiently small concentration of the precipitant was used to prevent denaturation, a part of the protein was not precipitated and the filtrate as well as the precipitate possessed growth-promoting properties. To afford some basis of comparison the method was adopted, therefore, of determining the concentration of nitrogen in the solution of the precipitate or the filtrate, and in the original extract, and then diluting the original extract with Tyrode solution so that it contained the same per cent of nitrogen as the experimental medium.

Tests on a number of precipitates were made in which the cultures were kept for only a few passages. In most cases, the tissue cultivated in the control extract and that in the solution of the protein precipitate grew at the same rate. No case occurred in which the precipitate showed any greater activity than the original extract. In some cases it was less active, because the method of fractionation either denatured the protein or destroyed some activating substance, for the activity of embryo juice is easily destroyed by heat, aging, and many chemical substances. Precipitation by alcohol, even at low temperature, always gave a precipitate less active than the original extract.

A fractionation of the protein by alcohol was carried out by adding a small amount of alcohol, centrifuging the precipitate, and then increasing the concentration of alcohol, etc., until ten fractions were obtained. The first three or four of these precipitates were quite active, the activity decreasing as the concentration of alcohol increased until practically no activity was obtained from the last fractions. The filtrate from precipitation by an equal volume of alcohol contains a considerable amount of active substance, also a considerable quantity of protein. A 50 per cent alcoholic solution will extract from embryo

ACTION ON FIBROBLASTS OF THE PROTEIN FRACTION OF EMBRYONIC TISSUE EXTRACT.

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It is known that various tissues will grow indefinitely *in vitro* in a medium consisting of one part of plasma and one part of embryonic tissue juice,¹ and that it is the embryonic tissue juice and not the plasma that furnishes the necessary substances for cell nutrition and multiplication.² Whether there is in embryonic extract a specific substance with a function of initiating or producing cell division, or whether the embryonic extract simply contains the essential nutrient substances required by the cells, is not known. Neither is there any knowledge concerning the chemical nature of the substances present in the extract which are utilized by the cells. The work reported in this paper is a preliminary examination of the action of the protein-containing fraction of embryonic extract in relation to its ability to promote the growth of fibroblasts in pure culture, or from heart tissue.

The protein of the embryonic tissue extract was precipitated in a variety of ways and redissolved in a volume of Tyrode solution equal to the volume of tissue juice from which the precipitate was obtained. It was found that the protein precipitate, provided it could be entirely freed from the reagent used for its precipitation and again brought into solution, contained some of the growth-stimulating action of the original extract. Ammonium salts, trichloroacetic acid, picric acid, pyridine, etc., were too difficult to remove and were toxic to the tissues. Carbon dioxide passed through a diluted solution, ethyl,

¹ Carrel, A., *J. Exp. Med.*, 1912, xv, 516; 1913, xvii, 14; 1914, xx, 1. Fischer, A., *J. Exp. Med.*, 1922, xxxv, 367. Ebeling, A. H., *J. Exp. Med.*, 1922, xxxv, 755. Carrel, A., and Ebeling A. H., *J. Exp. Med.*, 1923, xxxviii, 487. Ebeling, A. H., *J. Exp. Med.*, 1925, xli, 337.

² Carrel, A., and Ebeling, A. H., *J. Exp. Med.*, 1921, xxxiv, 317. Carrel, A., *J. Am. Med. Assn.*, 1924, lxxxii, 255; *Brit. Med. J.*, 1924, ii, 140.

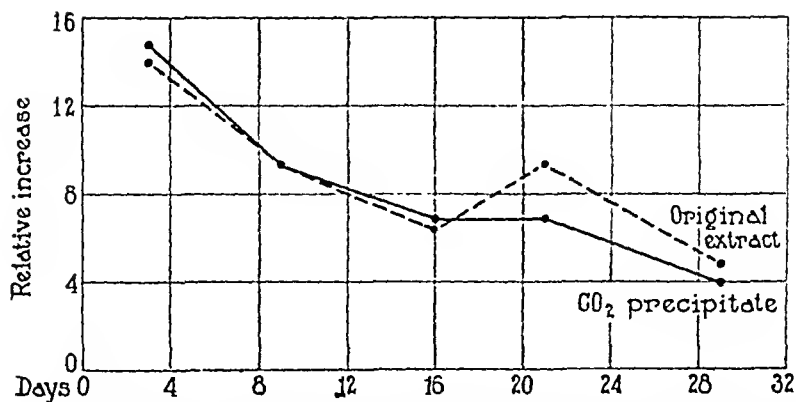


FIG. 1. Comparison of the rate of growth of fibroblasts in the protein precipitated by CO₂ and in the original extract diluted to the same nitrogen content.

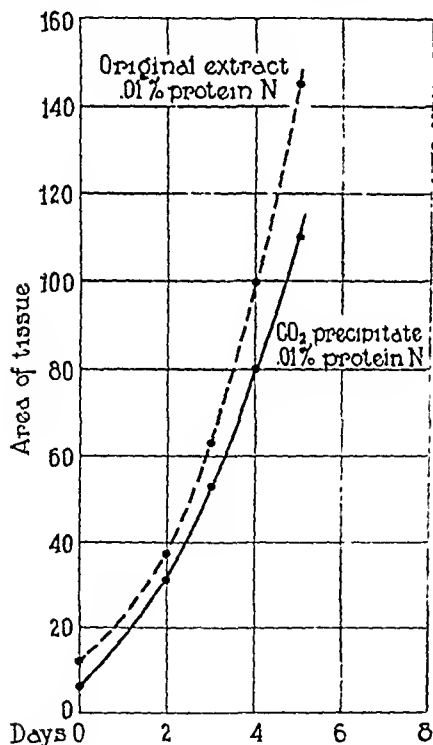


FIG. 2.

FIG. 2. Comparison of the rate of growth of fibroblasts from embryo heart in embryo extract and in the protein precipitated from it by CO₂.

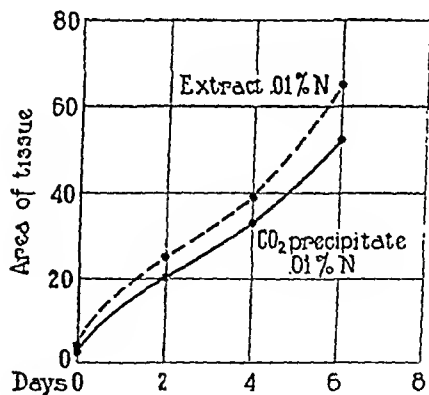


FIG. 3.

FIG. 3. Comparison of the rate of growth of a 13 year old strain of fibroblasts in embryo extract and in the protein precipitated by CO₂.

pulp an appreciable quantity of protein and produce an active extract when the alcohol is removed.

A few adsorption experiments were also tried, with charcoal, kaolin, and alumina as adsorbents. If a sufficient quantity was used to adsorb all the protein, the filtrate remaining was without any noticeable activity. If all the protein was not adsorbed, the filtrate generally exhibited some activity. The experiments tried on redissolving the active substances from the adsorbents were not successful.

These preliminary tests indicate that the protein of the extract is the essential nutritive substance upon which fibroblasts live *in vitro* and if, besides the protein, there is some specific substance essential for cell multiplication, it is united to the protein by a chemical or physical bond or is readily adsorbed on the protein as it precipitates.

Experiments on cultures in which the tissue is grown for only one passage of 2 or 3 days are valuable for preliminary information but are not conclusive, since it is quite probable that the tissue may carry along with it from its old medium, either mechanically or already adsorbed, some substance essential for its growth. The only convincing experiments, therefore, are those in which the tissues live for several passages in the experimental medium and its control. This is a time-consuming process, so as yet only results with the protein precipitated by carbon dioxide and its filtrate have been procured in this way. Tests have been made on the precipitate, using the method of small cultures in which the tissue is cut at every passage,³ and also that of cultivation in flasks⁴ where the tissue is not removed but the total accumulated growth over the entire period may be observed (Figs. 1 to 3). It is evident from the curves that the rate of growth of the tissue in the protein precipitated by CO₂ and redissolved in Tyrode is approximately the same as that in the original extract diluted to contain the same per cent of protein. The condition of the cells in the experimental medium was as good throughout as in the control. Since the experiment (Fig. 1) was carried on for 28 days, it seems conclusive that the protein precipitate contains all the necessary nutritive substances. Only a fraction of the protein, however, is precipi-

³ Ebeling, A. H., *J. Exp. Med.*, 1921, xxxiv, 231.

⁴ Carrel, A., *J. Exp. Med.*, 1923, xxxviii, 407.

stimulation of growth, causing greater migration of the cells, but they do not produce an increase in the mass of the tissue, as is done by the protein fraction of embryo juice. Further confirmation of these findings is reported in the following paper.

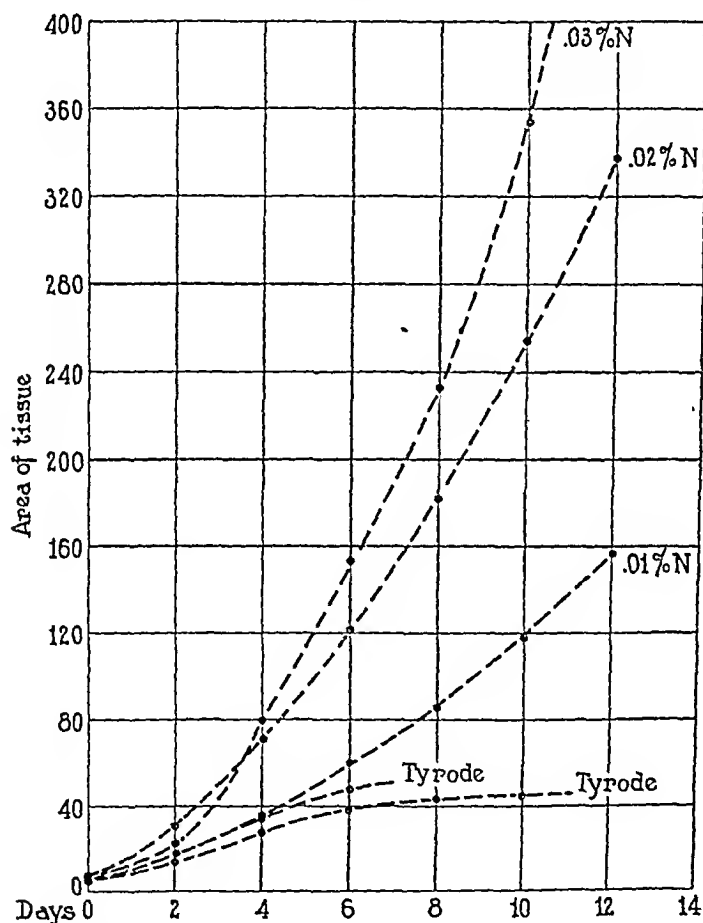


FIG. 5. Rate of growth of fibroblasts in embryo extract diluted to contain 0.01, 0.02, and 0.03 per cent of nitrogen. Note that the shape of the curve for media containing nutrient materials is quite different from that for Tyrode solution.

The rate of growth of fibroblasts is proportional to the concentration of active substances in the medium, a fact well illustrated by their growth in embryonic tissue extract at different dilutions. In the experiments recorded in Fig. 5, one sample of tissue juice was diluted so as to contain 0.01, 0.02, and 0.03 per cent nitrogen. Tissues do not,

tated by CO_2 , approximately one-half of that originally present. The filtrate is also quite active (Fig. 4), and relatively in proportion to its protein content. A careful examination of the curves for the CO_2 precipitate and the CO_2 filtrate shows that there is a little activity due to the non-protein nitrogen present. This causes a slightly increased

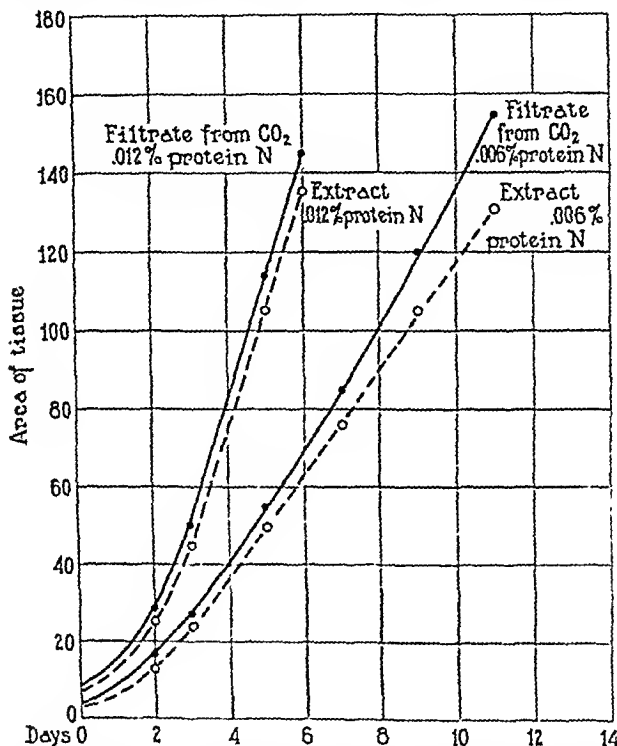


FIG. 4. Comparison of the rate of growth of fibroblasts from embryo heart in embryo extract and in the CO_2 filtrate containing equal concentrations of protein nitrogen.

area in the growth of tissue in the extract over that in the CO_2 precipitate, which contains only protein nitrogen and a slightly greater area in the CO_2 filtrate than in the diluted extract. The basis of comparison here was an equal content of protein nitrogen. As has been shown previously⁵ amino acids are capable of producing a slight

⁵ Carrel, A., and Ebeling, A. H., *Compt. rend. Soc. biol.*, 1924, xc, 31.

purification so as not to be biologically the same, even if it appears to have the same chemical properties.

The protein of embryonic tissue juice has been tested chemically and found to be a mixture of nucleoprotein and a glycoprotein with mucin-like properties. However, when these proteins were isolated in a comparatively pure state from embryo pulp and tested on growing tissues, they did not seem to possess any growth-promoting action.

Pure sodium nucleate from embryonic pulp was prepared and found to be inactive, as were many other substances also, among which were egg albumin, egg globulin, crystalline egg albumin, nuclealbumin, nucleoglobulin, lecitho-albumin, thymus nucleic acid, and acid meta-protein from crystalline egg albumin. Crystalline egg albumin prepared with ammonium sulfate proved to be toxic even after prolonged dialysis, but that from which the ammonium sulfate was removed by recrystallizing three times with Na and K sulfates, and washing with sodium chloride and acetic acid, was not toxic. A few of the above preparations appeared to be slightly stimulating in the first passage but proved inhibiting after three passages, and in no case was there any growth as large as that obtained with even a very small quantity of embryo tissue extract.

Since the protein precipitated by almost any method carries along with it by adsorption some substances of lipoid nature, these precipitates and the original extract were purified by extraction with ether. As will be reported in another paper of this series, the growth-promoting action was not lost (Fig. 6). Therefore, it is the protein, and not the lipoid associated with it, that carries the activity of the embryo tissue extract.

DISCUSSION AND SUMMARY.

The above experiments indicate that the growth-stimulating substance found in embryonic tissue extract, which has been responsible for the continuous growth of fibroblasts *in vitro* for 14 years, is either protein in nature or closely associated with the protein of the extract and adsorbed by it. If any specific hormone responsible for cell division is present, it is united to the protein or carried along with it in its first precipitation. It seems probable that the tissues utilize this protein for the nitrogen which they build into protoplasm. Whether it

however, grow at the same rate in different preparations of extracts, even when they contain the same per cent of protein. Curves such as those figured are, therefore, truly indicative of the concentration of growth-activating substances in the experimental medium when compared with a sample of the original extract from which the experimental medium was prepared.

Since the protein of the embryonic tissue extract appears to contain the essential nutritive and activating substances, experiments were undertaken to purify it by the process of repeated precipitation. This

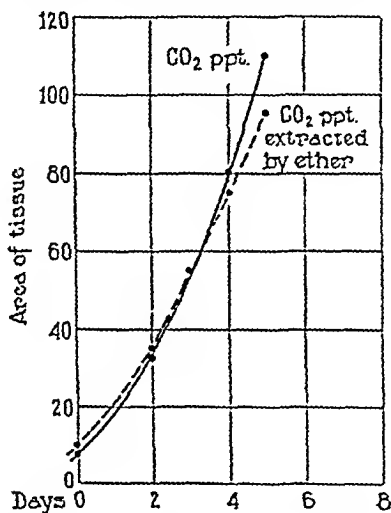


FIG. 6. Comparison of the rate of growth of tissue in the CO₂ precipitate and the CO₂ precipitate extracted by ether.

was tried with the precipitates obtained by carbon dioxide, acetic acid, and alcohol. The precipitation was carried out at low temperature and, if only a part of the total protein was precipitated, it was dissolved in a correspondingly smaller volume of Tyrode solution before being reprecipitated. The activity of the first and third precipitates was tested. The third precipitate was found to be considerably less active in each case; in fact, when it was compared with Tyrode solution, it was found to be almost inactive. Obviously, the protein has either lost some substance attached to it which was essential to the growth of the tissues or it has itself been altered in the process of

is first hydrolyzed before adsorption by the tissues has not been ascertained as yet. It has been shown in other experiments reported in the following paper that the amino acids of the tissue juice do not suffice for the growth of fibroblasts and that hydrolyzed tissue juice is toxic in the same way that a too concentrated mixture of amino acids is toxic.

The results of the foregoing experiments may be summarized as follows:

1. Fractionation of embryo tissue juice has shown that it is the protein fraction that contains the activating substance.

2. Tissues continue to grow for a long time in the protein of the extract precipitated by CO_2 and at a rate approximately equal to that in the original extract diluted to the same nitrogen concentration.

3. The non-protein nitrogen gives slight stimulation to growth.

4. Purification of the protein by repeated precipitation destroys its growth-promoting properties, but whether this is due to a denaturing of the protein,—which occurs very readily,—or to loss of some substance possibly an enzyme attached to it, has not been ascertained.

5. Preparations of purified proteins from embryonic tissue and egg white have shown no marked nutritive or stimulating action. A number of other pure substances have been tried without effect.

The authors wish to express their thanks to Dr. Michael Heidelberger for his criticisms and suggestions in connection with this work.

not been ascertained by the experiments thus far conducted *in vitro*. Extensive experiments carried out in this laboratory with many individual amino acids and also with mixtures have shown that the acids stimulate the tissues and produce a greater area of migration, but that they do not prolong the life of the tissues and in themselves are not sufficient to bring about continued multiplication of cells.⁴ On the other hand, embryonic tissue juice may contain certain specific acids essential to the growth of the tissues which are lacking in serum and in artificial mixtures of amino acids.

The study of the effects of the amino acids and dialyzable constituents of embryonic tissue juice was carried out in several ways.

1. The embryonic tissue extract was dialyzed in very permeable collodion sacks until free from amino acids, and compared with the original extract for its growth-promoting properties.

2. The dialyzed extract was tested to ascertain whether an enzyme were present which produced an increase in the amino acids after those originally present were removed.

3. The amino acids were separated from the extract by ultrafiltration along with other ultrafilterable constituents, and their action was tested for their growth-promoting effect.

4. An artificial mixture of sixteen amino acids was added to extract dialyzed until free from amino acids, and this was compared with the dialyzed extract.

5. The ultrafiltrate was added to extract from which the acids had been removed by dialysis and this was compared with the extract free from amino acids.

6. The amino acids obtained by hydrolyzing embryonic tissue juice, both by acid and by trypsin, were tested at various concentrations for growth-promoting action.

The technique of Carrel and Ebeling⁵ for the cultivation of the tissues was used, the two halves of the same culture being carried through several passages, one in the experimental medium and one in the control medium. Measurements of the area of growth were made every 2 days.

The Action of Embryonic Extract, Dialyzed until Free from Amino Acids, on the Growth of Fibroblasts.

In order to remove the amino acids completely as rapidly as possible from the extract, collodion bags of high permeability were used.

⁴ Carrel, A., and Ebeling, A. H., *Compt. rend. Soc. biol.*, 1924, xc, 31.

⁵ Carrel, A., *Compt. rend. Soc. biol.*, 1923, lxxxix, 1017. Ebeling, A. H., *J. Exp. Med.*, 1921, xxxix, 231.

EFFECT OF THE AMINO ACIDS AND DIALYZABLE CONSTITUENTS OF EMBRYONIC TISSUE JUICE ON THE GROWTH OF FIBROBLASTS.

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It is known that embryonic tissue juice is essential to the continuous growth of connective and epithelial tissues *in vitro*;¹ but the nature of the chemical constituent or constituents present in the proteins of the embryonic tissue extract,² which give to it its peculiar growth-promoting properties, has not been discovered. This paper will deal with the separation of the extract into its dialyzable and non-dialyzable constituents and the action of each on the growth of fibroblasts *in vitro* with special reference to the amino acid content of the two fractions. The amino acids were separated from the rest of the extract in order that evidence might be obtained as to whether the nitrogen required for the synthesis of protoplasm is utilized in the form of amino acids.

Since the proteins upon which animals subsist are broken down into amino acids before entering the blood stream and since animals may be maintained in nitrogen equilibrium on enzyme digests of proteins consisting almost entirely of amino acids, it would seem quite probable that the tissues utilize nitrogen in this form. It has been found that the amino acids of serum are incapable of supporting the growth of fibroblasts in pure culture,³ but whether or not this is true *in vivo*, where the circulating blood brings constantly renewed supplies, has

¹ Carrel, A., *J. Exp. Med.*, 1912, xv, 516; 1913, xvii, 14; 1914, xx, 1. Fischer, A., *J. Exp. Med.*, 1922, xxxv, 367. Ebeling, A. H., *J. Exp. Med.*, 1922, xxxv, 755. Carrel, A., and Ebeling, A. H., *J. Exp. Med.*, 1923, xxxviii, 487. Ebeling, A. H., *J. Exp. Med.*, 1925, xli, 337.

² Baker, L. E., and Carrel, A., *J. Exp. Med.*, 1926, xlv, 387.

³ Carrel, A., and Ebeling, A. H., *J. Exp. Med.*, 1923, xxxvii, 759.

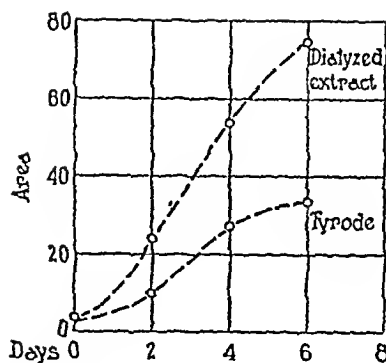


FIG. 1. Rate of growth of fibroblasts from embryo heart in embryo tissue juice dialyzed free from amino acids. Compared with growth in Tyrode solution. Cultures in flasks.

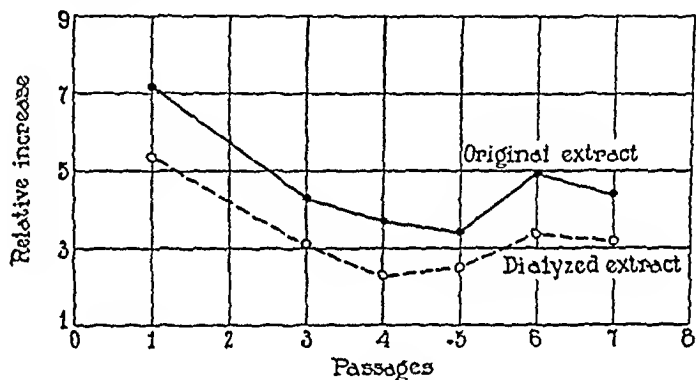


FIG. 2. Comparison of the growth of an old strain of fibroblasts in embryo extract and in the same extract dialyzed free from amino acids. Concentration of nitrogen is the same in each case.

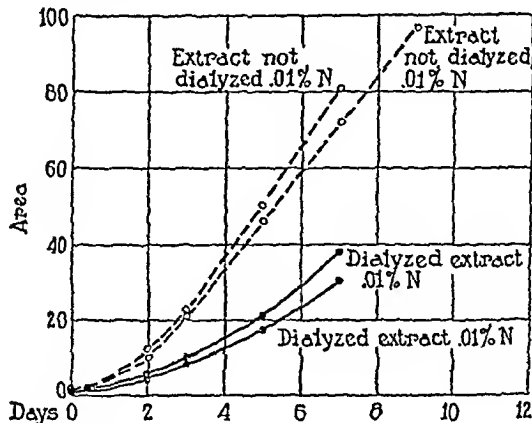


FIG. 3. Comparison of the rate of growth of fibroblasts in embryo tissue extract and in extract dialyzed free from amino acids. Cultures in flasks.

The bags were prepared according to the technique of Gates,⁶ on gelatin capsules to which a glass neck had been sealed. The capsules were dipped once in a thick collodion solution, drained, and exposed to the air for 2 minutes, then immersed in 95 per cent alcohol for 10 minutes, and hardened in cold water. After the gelatin was washed out with hot water, they were preserved in 95 per cent alcohol until desired for use, at which time the alcohol was washed out with sterile water and the bag itself subjected to dialysis until all traces of alcohol were removed. Tissue extract dialyzed in these sacks became free from amino acids in 20 to 30 hours. Determinations of amino nitrogen were made from time to time in the Van Slyke micro apparatus, until a minimum amount of amino nitrogen remained, *i.e.*, only the quantity characteristic of the protein itself as separated from the extract by precipitation methods. In a number of the experiments, running water was circulated in a narrow coil immersed in ice before coming into contact with the dialysis membrane. In others, tap water at 12–15°C. was used.

The dialyzed extracts were made isotonic either by the addition of an equal volume of double strength Tyrode solution or by evaporating to dryness and redissolving in ordinary Tyrode solution. In either case, the concentration of protein nitrogen was determined in the dialyzed extract and in a sample of the original extract saved as a control, and dilutions were made to bring the two to the same per cent of protein. The pH was also adjusted to the same value, approximately 7.6.

All the samples of dialyzed extract, in spite of differences in details of preparation, gave the same result when tested on growing tissues. The growth-stimulating substance was not entirely removed, for the rate of growth was considerably greater in the dialyzed extract than in Tyrode solution (Fig. 1). A very much smaller area of growth was obtained in the dialyzed extract than in the original one (Figs. 2 and 3). This difference, however, may be attributed to two causes: the loss of the amino acids and other dialyzable constituents, or to a denaturation of part of the protein through the long continued action of water. The dialyzed extract often exhibited a greater opalescence than the original one, especially in reflected light, indicating a change in the physicochemical state of the protein. Such preparations gave very poor growth. Others showed a much smaller decrease in growth-promoting properties, both the experiment and control remaining in good condition.

Since it is conceivable that the extract dialyzed until free from

⁶ Gates, F. L., *J. Exp. Med.*, 1921, xxxiii, 25.

slightly larger (Table I). The behavior was quite analogous to that of tissues in artificial mixtures of amino acids reported by Carrel and Ebeling.⁴ When 10 per cent of fresh embryonic extract was added to the ultrafiltrate and also to the Tyrode solution, in order to prolong the life of the tissues, it was found that a larger area of growth was obtained in the presence of the ultrafiltrate at every passage (Fig. 4), although the difference was not sufficient to increase the mass of the tissues in one case more than in the other. It is, therefore, only an increase in the area of migration and not in the mass of tissue that

TABLE I.
Rate of Growth of Fibroblasts in the Ultrafiltrate of Embryo Juice.
Tyrode Solution Used as Control.

Group No.*	Preparation No.	Rate of growth in		Ratio: E/C.	Remarks.
		Tyrode (C).	Ultrafiltrate (E).		
6304	X-3	5.93	8.02	1.35	1st passage.
6312	X-3	5.85	7.90	1.35	2nd "
6327	X-3	5.57	8.20	1.47	3rd "
6276	X-3	4.03	5.85	1.45	1st "
6314	X-6	8.8	9.6	1.09	1st "
6328	X-6	5.27	7.76	1.47	2nd "
33268	X-26	4.88	5.2	1.06	1st "
33286	X-26	5.0	5.0	1.0	2nd "
33297	X-26	4.71	5.95	1.26	3rd "
Average.....				1.28	

* Each group consists of three or four experiments.

is produced by the amino acids and other ultrafilterable constituents of embryo tissue juice.

The residue from the ultrafiltrate retained some growth-stimulating action, but so much of the protein became insoluble in the process that it was not a valuable experimental material.

Effect of Adding the Ultrafiltrate or an Artificial Mixture of Amino Acids to Dialyzed Extract.

Since the removal of amino acids from tissue extract by dialysis diminishes its growth-promoting action, an experiment was tried to

amino acids might not remain so, owing to the action of enzymes on its protein, the dialyzed extract was tested at various pH values for a regeneration of its amino acids. The increase of amino nitrogen was so slow that growth of tissues in this dialyzed extract could not be attributed to that cause. Therefore, it appears that the nitrogenous substances utilized by the fibroblasts growing in dialyzed extract are other than amino acids.

The Action of the Amino Acid Fraction of Tissue Juice Obtained by Ultrafiltration.

It was found that the amino acids of the tissue extract could be separated from the protein by a process of ultrafiltration under pres-

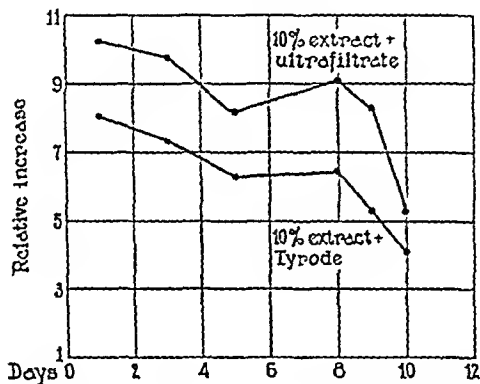


FIG. 4. Effect of the ultrafilterable constituents of embryo tissue juice on the rate of growth of fibroblasts.

sure through a collodion sack prepared exactly as those used for the dialysis. No protein passed through these sacks, *i.e.*, the ultrafiltrate gave none of the tests for protein, and there was no increase in amino nitrogen after hydrolysis by acid. The amino nitrogen content of the ultrafiltrate was found to be almost equal to that of the original tissue juice, the difference being due to amino groups attached to the protein molecule. Experiments on growing fibroblasts, with this ultrafiltrate, demonstrated that the tissues lived no longer in it than in Tyrode solution, although the area of growth at each passage was

for when this same dialyzed extract containing the ultrafiltrate was compared with a sample of the original extract not treated in any way, the growth in the original extract was somewhat better (Fig. 6). This phenomenon is probably not due to any difference in the amino acids of the embryonic extract, but to a denaturing of part of the protein by the action of water, and by changes in temperature and concentration.

Finally, in order to ascertain whether the amino acids or some other ultrafilterable and dialyzable constituents were responsible for the effect observed, experiments were made in which an artificial mixture of sixteen amino acids was added to the dialyzed extract. The results

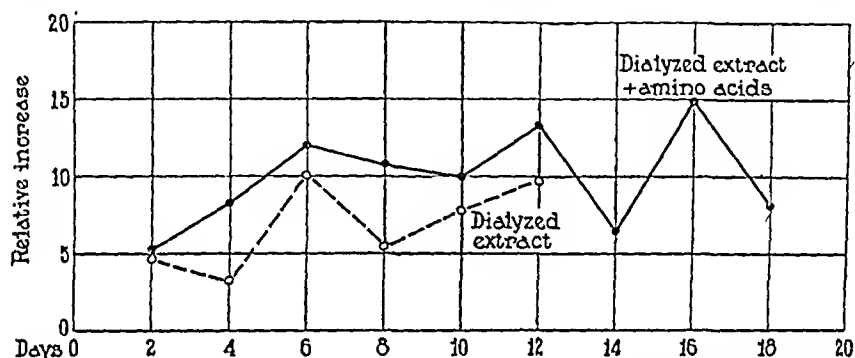


FIG. 7. Effect of the addition of an artificial mixture of amino acids to dialyzed embryo extract on the rate of growth of fibroblasts.

were quite analogous to those obtained on adding the ultrafiltrate (Fig. 7). In all cases, the presence of the amino acids or ultrafiltrate gave a larger area of migration at each passage, but this did not bring about any increase in the mass of the tissue.

Action of Amino Acids Obtained by Hydrolysing Embryonic Tissue Juice by Acid and by Trypsin.

Since the concentration of amino acids in the embryonic extract and in the ultrafiltrate is quite small (6 to 12 mg. per 100 cc.), it was thought that a higher concentration of the acids obtained by the hydrolysis of the tissue juice protein with acid or trypsin might produce decided growth, although those already present had failed to do so. These digests, however, proved toxic even when diluted to such an

see whether the addition of the amino acids and other dialyzable constituents obtained by ultrafiltration would restore this action. Fig. 5 illustrates the rate of growth of tissues in the dialyzed extract.

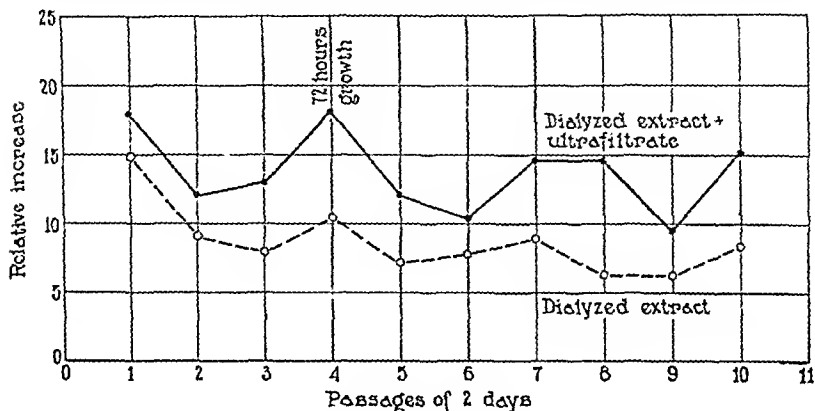


FIG. 5. Effect of replacing the constituents removed by dialysis with the ultrafiltrate of embryo tissue juice on the rate of growth of fibroblasts.

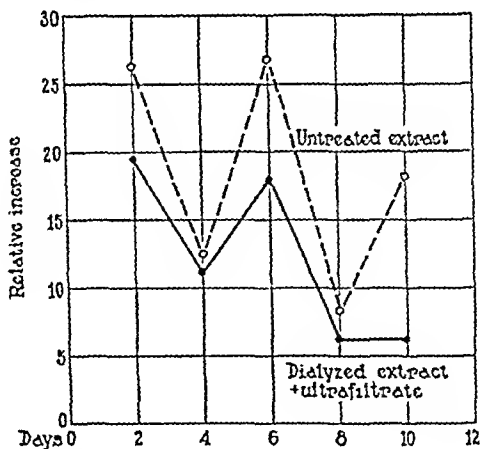


FIG. 6. Comparison of the rate of growth of fibroblasts in dialyzed extract plus the ultrafiltrate with that in the original extract.

and in the dialyzed extract to which the ultrafiltrate has been added. It appears that some of the lost activity is restored, for there is a slight increase in the migration of the cells. It is not all restored however,

It is probable that the amino acids are not the form in which tissues absorb the nitrogen which they build into protoplasm. These conclusions are in accord with previous experiments in which the growth of tissues, in protein precipitates of embryonic extract containing no uncombined amino acids, was studied.² The amino acids of the extract together with other ultrafilterable substances are unable to maintain the life of tissues for a longer time than does Tyrode solution. They do stimulate cell migration and possibly multiplication. This effect is probably not due to some other diffusible substance which might be mixed with the amino acids, because a similar stimulation was observed when a solution of sixteen amino acids (given to us by Dr. P. A. Levene, or obtained from Hoffmann La Roche and Pfanstiehl) was added to the medium of fibroblasts. The stimulation of mitosis, observed by Wright⁷ in embryonic cells cultivated in a diffusate of embryonic tissue juice, is probably a phenomenon of the same nature. Amino acids presumably play an important rôle in cell life since a greater area of tissue was invariably obtained in their presence, although its mass was not noticeably increased. Even if they cause only a temporary activation of cell metabolism, this may be a very important factor in normal and pathological phenomena. A possible explanation of these phenomena may rest on the hypothesis that some of these acids are utilized as food when other necessary substances with which they can unite are supplied by the protein fraction of the embryo juice, although alone they cannot be utilized. It is also conceivable that, with a different method of cultivating tissues, the cumulative effect of this greater migration might be observed over a longer period of time. It must be remembered that these experiments do not mean that tissues can grow in the complete absence of amino acids, since some amino acids are always present in the plasma which forms part of the medium, but merely that the growth-promoting substances, which distinguish embryonic juice from other fluids in its capacity to maintain the life of fibroblasts and epithelial cells indefinitely *in vitro*, are not to be found among its dialyzable or ultrafilterable components.

⁷ Wright, G. P., *J. Exp. Med.*, 1926, xliii, 591.

extent that they contained very little more amino nitrogen than an ordinary embryonic extract (Table II).

TABLE II.

Rate of Growth of Fibroblasts in Amino Acids Obtained by Hydrolysis of Embryo Juice Protein.

Tyrode Solution Used as Control.

Group No.*	Preparation No.	Rate of growth in		Ratio: E/C.	Remarks.	
		Tyrode (C).	Ultrafiltrate (E).			
34-144 C	X-69	9.1	7.5	0.82	1st	Passage. { Hydrolyzed by acid. Tryptophane and cysteine added.
9 C	X-69	12.0	9.7	0.81	2nd	
20 C	X-69	10.7	8.0	0.75	3rd	
34 C	X-69	2.8	1.0	0.36	4th	
8 C	X-70	11.1	7.2	0.65	1st	{ Hydrolyzed by acid.
21 C	X-70	8.45	4.71	0.56	2nd	
35 C	X-70	4.0	1.95	0.49	3rd	
212 C	X-83	12.0	15.0	1.25	1st	{ Hydrolyzed by tryp- sin. 11.5 mg. amino N per 100 cc.
224 C	X-83	9.2	8.8	0.96	2nd	
235 C	X-83	10.5	3.9	0.37	3rd	
23 C	X-72	10.9	9.8	0.90	1st	{ Hydrolyzed by tryp- sin. 62 mg. amino N per 100 cc.
36 C	X-72	4.13	2.8	0.68	2nd	
47 C	X-72	12.2	5.1	0.42	3rd	
205 C	X-82	15.2	18.3	1.2	1st	{ Hydrolyzed by tryp- sin. 6 mg. amino N per 100 cc.
217 C	X-82	10.5	11.4	1.08	2nd	
225 C	X-82	12.6	8.78	0.70	3rd	
236 C	X-82	5.1	3.1	0.61	4th	

* Each group consists of three or four experiments.

DISCUSSION AND CONCLUSIONS.

The above evidence indicates that the substance or substances present in embryonic tissue juice which give it its peculiar growth-promoting action are not removed by dialysis or by ultrafiltration in collodion bags of high permeability, and that they are not of the nature of amino acids.

SUMMARY.

The ultrafilterable constituents of embryonic tissue extract are unable to support cell life *in vitro*. They stimulate cell migration and possibly multiplication, without increasing the mass of the tissue.

Embryonic tissue extract freed from amino acids by dialysis still retains a considerable part of its growth-promoting properties.

The area of growth of tissues in embryonic tissue extract free from amino acids is appreciably less than that with the whole extract, probably owing to the denaturation of part of the protein, or perhaps to the inactivation or loss of an enzyme.

The addition of either the ultrafilterable components or an artificial mixture of amino acids to this dialyzed extract increases the area of cell migration but does not restore all the activity lost on dialysis.

The observed differences in growth of tissue, due to the addition or removal of dialyzable and ultrafilterable constituents of the extract, prove that the amino acids produce a more active cell migration and possibly multiplication, but no building up of new protoplasm.

The authors wish to express their thanks to Dr. Michael Heidelberger for his criticisms and suggestions in connection with this work.

the greater or lesser virulence of the parasites, or to greater or lesser susceptibility of the host. If the latter is the case, what factors are responsible for the seasonal variation?

Recent experimental studies indicate a seasonal variation in the susceptibility of animals to certain diseases other than malaria. Brown and his associates (4) have shown that there is a seasonal rhythm in the malignancy of a transplanted tumor in rabbits, which the authors attribute to fluctuations in the resistance of the animals rather than to variations in the malignancy of the tumor. Pritchett (5) has shown a similar seasonal fluctuation in experimental mouse typhoid. The mortality among infected animals showed a distinct seasonal variation, being high in the spring and fall and low during the summer.

The causative factor in this seasonal variation of malignancy is not known. Lenz (6) attributes the increase of malaria relapses to sunlight, and puts forward the hypothesis that the sunlight stimulates parthogenesis of the gametocytes. There is no evidence in support of his hypothesis. Reinhard (7) presents evidence on the provocation of malaria relapses by ultra-violet light. Similar results are reported by Whitmore (8) in bird malaria. Brown and his coworkers (4) correlate the malignancy of the transplantable rabbit tumor with meteorological factors, low malignancy being associated with periods of maximum and minimum sunlight, while high malignancy occurred at times of sudden changes in sunlight both in the spring and autumn. Pearce and Van Allen (9) tested this observed correlation experimentally, and reported confirmative evidence. They found that animals kept in constant darkness showed a slight but distinct increase in the resistance to the tumor, while the effect under conditions of constant light was even more marked, the disease assuming a milder form.

It appears from the above that among the various environmental factors influencing host resistance light plays a significant rôle. But there are other environmental factors, among them heat and cold, dryness and moisture which may also play a part in determining host resistance. In this paper we present experimental data on the effect of light, darkness, and moisture on the resistance of guinea pigs to trypanosome infections.

Materials.

The strain of trypanosome used was *Tr. evansi* isolated from infected mules in 1923. Since its isolation this strain has been maintained in guinea pigs, and at present its virulence and pathological effects are quite constant. The strain invariably produces a fatal infection in guinea pigs and rabbits. In guinea pigs of 250 to 450 gm. the incubation period is quite constant, varying from 5 to 8 days with an average of 6.2 days. (Among 52 normal animals observed during 1925, the incubation periods were: six, 8 days; eleven, 7 days; twenty-two, 6 days; and

SUSCEPTIBILITY AND RESISTANCE TO TRYPANOSOME INFECTION.

II. THE RELATION OF PHYSICAL ENVIRONMENT TO HOST SUSCEPTIBILITY TO INFECTION.

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INTRODUCTION.

In this series of investigations, we set out to examine the relation of various factors to the resistance or susceptibility of experimental animals to infection with a protozoan—the trypanosome. In previous communications (1, 2) we have reported data bearing on the acquired susceptibility and resistance resulting from the interaction of the parasite and host. In this paper we present our observations on the effect of external environmental factors, as for example, light and moisture, on the susceptibility of the host to the infection and on the severity of the disease imposed.

Recent experimental epidemiologic studies by Webster (3) indicate that the virulence of a given strain of pathogenic organisms is constant. In that case variations in malignancy and host susceptibility can only be accounted for by assuming that the resistance of the host is variable, and is modified from time to time by a variety of conditions. Since many diseases have a seasonal character, this variation in resistance must have a seasonal rhythm.

Seasonal variations in the incidence and intensity of malaria outbreaks are noted everywhere. The seasonal occurrence of malaria epidemics is due to the optimum conditions for the development of the anopheline vectors. But, the relatively high prevalence of relapses with the advent of the spring and the occurrence of benign tertian malaria in the summer and of malignant tertian malaria in the fall still baffle the malariologists and await explanation. The questions still unsolved are: Whether the difference in the observed malignancy is due to

EXPERIMENTAL.

Preliminary experiments were made in order to ascertain the effects of the exposure under the various conditions of the experiments on the temperature, white cell, and differential counts of the animals. The results of these preliminary experiments are of interest in that they indicate that profound changes of greater or lesser duration may be produced by relatively short periods of exposure.

Exposure to Direct Sunlight.

An area of 4 to 5 cm. square was shaved on the back of animals which were then placed in a battery jar, or a wooden box. The jar or box was so inclined that the sun rays played constantly on the shaved area. In later experiments the animals were tied on an inclined board, and covered with a heavy towel, leaving only the shaved area exposed to the sun's rays. The exposure was always made between 9 and 9.30 a.m. with the sky and atmosphere clear. The facts must be emphasized that the experiments were carried out in Palestine, a region where the sunlight is of such great intensity that exposure of the guinea pig for but an hour may cause its death.

The exposure under water was carried out by the second of the methods described. The animals were tied to a board set obliquely in a tin box, made especially for the purpose, in such wise that the heads of the animals were outside the box. The heads were covered with a moist towel, and the box filled with water so that the shaved surface was 1 to 1.5 cm. under water.

After the exposure, the animals were placed in dry boxes in the animal room, and kept under the usual light conditions.

The effects of these exposures are illustrated in Tables I to III. An exposure of 15 minutes to direct sunlight raises the rectal temperature 1.5°C . or more, and changes the leucocytic formula. The temperature effect is temporary, lasting about 2 hours, but the blood cell change is more enduring, and recovery is very slow.

Insolation through a water bath produces none of these changes. The rectal temperature falls about 2.5°C ., the blood cell formula remains unchanged, and there is an increase in the total leucocyte count. Even the infection, which is usually accompanied by a depression of the polynuclears prior to the invasion of the circulation, does not produce this effect in water-immersed animals until several days after the trypanosomes invade the circulation.

thirteen, 5 days.) The intensity of the infection as measured by the frequency of blood invasion also appears to be fairly constant. The duration of illness (or life) shows a tendency to seasonal fluctuations, a fact that will be discussed later.

Variations in the dosage of trypanosomes were not important within the limits used. Our dose was 0.1 cc. of citrated infected blood, diluted to contain an average of one organism per microscopic field, injected into the peritoneal cavity. In some of the experiments a counted number of organisms were injected (in one experiment 75 and in another 400), but no differences in the results were observed.

In so far as possible, guinea pigs of the same weight were used for each experiment.

Methods of Study.

The details of the methods used in each type of experiment will be described below. In general the animals used in a given experiment were first examined and then subjected to the conditions of the experiment for some days prior to the infection. The changes due to the infection as such could, therefore, be distinguished from those produced by the exposure to light, darkness, or moisture.

The difficulty in these experiments was to select a suitable criterion for decreased or increased susceptibility. The variations in individual animals render it difficult to rely on a single pathological manifestation. The length of the incubation period is the most constant phenomenon; but the frequency and intensity of the blood invasion and the duration of the illness are also of value. Consequently, these three have been employed as indices of the changes in host susceptibility produced by the exposure under the experimental conditions. Normal animals kept under the usual light conditions served as controls.

The first objective was to ascertain whether exposure to one or another of the conditions to be tested produced a measurable change in the host susceptibility. The experiments were wholly qualitative in character and the procedure fairly simple. Guinea pigs were exposed for various lengths of time to the action of direct sunlight and an equal number of comparable controls were kept in total darkness, or under ordinary light conditions. Under the conditions of the experiments sun and heat action were not separable, and in order to eliminate the heat factor, sets of animals were exposed to direct sunlight while immersed in water baths.

The preliminary experiments showed that exposure to the conditions described, even though of short duration, produced appreciable

TABLE II.
Effect of Insolation through Water on Normal Guinea Pigs.

Time of exposure 15 min.					
	Before exposure.	After exposure, hrs.			
		0	2	4	24
Temperature.....	38.9°C.	36.2°C.	38.3°C.	38.6°C.	38.9°C.
Total white cell count.....	9,800		10,600	12,000	11,000
Polynuclears.....	58%		56%	56%	59%
Lymphocytes.....	36 "		38 "	39 "	35 "
Large mononuclears.....	6 "		6 "	5 "	6 "

TABLE III.
Effect of Insolation, Direct and through Water, on the Blood Picture and Blood Infection.

Time of exposure 15 min.															
After infection.	Direct.					Through water.					Control.				
	Total differential count.				Tr. ¹	Total differential count.				Tr. ¹	Total differential count.				Tr. ¹
	W.c.c.	P.	L.	M.		W.c.c.	P.	L.	M.		W.c.c.	P.	L.	M.	
days															
1	9,600	50	45	5	0	12,800	56	39	5	0					
2	8,900	47	49	4	0	14,000	54	40	6	1:5	8,000	50	45	5	0
4	8,200	45	50	5	1:10	14,800	52	44	4	3:1	7,900	45	51	4	0
5	7,200	43	52	5	1:5										0
6						11,000	48	48	4	2:1	7,000	41	54	5	1:5
7						12,400	47	47	6	6:1					

¹ Number of trypanosomes per microscopic field.

changes in the course of the infection. Exposure to direct sunlight for 15 minutes shortened the incubation period to 3 days instead of the 6 of the controls, while in the animals insolated under water for

TABLE I.
Effect of Insolation on Normal Guinea Pigs.

Time of exposure 15 min. daily.

	Before exposure.	After exposure, days.						Increase on exposure.
		0	1	3	6	8	10	
Temperature.....	38.3°C.	39.9°C.	38.5°C.					1.5°C.
Total white cell count..	10,600		9,600	8,900	9,000	8,880	9,600	
Polymorphonuclears.....	57%		48%	45%	47%	50%	51%	
Lymphocytes.....	37 "		47 "	49 "	47 "	45 "	46 "	
Large mononuclears.....	6 "		5 "	6 "	6 "	5 "	3 "	

Effect of 1 hr.'s exposure every day.

	Before exposure.	1	2	3	4	5	6
Total white cell count..	8,800	8,600	8,800	6,400	7,900	9,000	
Polymorphonuclears.....	64%	56%	21%	40%	60%	55%	
Lymphocytes.....	35 "	42 "	78 "	58 "	38 "	43 "	
Large mononuclears.....	1 "	2 "	1 "	2 "	2 "	2 "	

Effect of $\frac{1}{2}$ hr.'s exposure once.

	Before exposure.	1	3	4	5
Total white cell count.....	9,000		6,800	7,800	8,400
Polymorphonuclears.....	58%	40%	54%	51%	57%
Lymphocytes.....	39 "	58 "	42 "	46 "	41 "
Large mononuclears.....	3 "	2 "	4 "	3 "	2 "

Effect of 1 hr.'s exposure once.

	Before exposure.	1	2	3
Total white cell count.....	8,600	9,200	8,000	7,800
Polymorphonuclears.....	57%	36%	45%	47%
Lymphocytes.....	40 "	61 "	53 "	50 "
Large mononuclears.....	3 "	3 "	2 "	3 "

clearly that the animals kept in ordinary diffused light and especially those kept in total darkness were more resistant to the infection than those exposed for brief periods daily to the direct action of sunlight.

We have made some attempts to gauge the intensity of the sun rays by the Clark method (10). The first standard furnished through the kindness of Dr. Clark was not satisfactory. The second was more so, but it was received after our experiments were well under way. A comparison of intensities was not possible therefore. With the second standard an 8 minute exposure equalled one lithophone unit (50 per cent reduction).

The peculiar effect of immersion on the course of infection, in the absence of any change in the leucocytic ratio, led us to test the influ-

TABLE V.

Effect of Immersion of Animals for Short Periods in Water on Their Resistance to Trypanosome Infections.

No. of animals.	Time immersed.	Incubation period.	Frequency of blood invasion.	Duration of life.	Remarks.
	<i>min.</i>	<i>days</i>	<i>per cent</i>	<i>days</i>	
5	15	3.4	86	54	Both sets of animals were kept under the same conditions except for the immersion in water.
5	0	6	63	82	

ence of immersion for short periods without radiation. The results were practically identical with those observed in radiated immersed animals. It appears, therefore, that immersion as such produces a profound change in the resistance of guinea pigs to trypanosome infections. This has been noted in a number of experiments and cannot, therefore, be considered accidental.

It remains to be determined whether the effects produced by short exposures to direct sunlight are due to heat or specific rays and whether those produced by immersion are due to chilling or excessive moisture of the skin. It is clear, however, that the exposure of infected animals for brief periods daily causes significant changes in the course of the infection.

The lowering of resistance to trypanosomes which follows upon

the same period, the incubation period was only 2 days, and there was a prompt and heavy invasion of the blood stream.

The duration and time of exposure make an appreciable change in the results in animals infected with the trypanosome. A single exposure of half an hour on the day of infection reduces the incubation period, and shortens the duration of the illness by about half. A longer exposure, 1 hour or more, may, as already stated, cause sudden death with acute symptoms: the exposed area is inflamed, there is severe hemorrhage in the peritoneal cavity, the coagulability of the

TABLE IV.

Effect of Insolation on the Course of Trypanosome Infection in Guinea Pigs.

(a) Insolated directly.						(a) Insolated through water.					
No. of animals.	Length of exposure before infection.	Time of exposure.	Average incubation period.	Frequency of blood invasion.	Average duration of illness.	No. of animals.	Length of exposure before infection.	Time of exposure.	Average incubation period.	Frequency of blood invasion.	Average duration of illness.
	days	min.	days	per cent	days		days	min.	days	per cent	days
2	2	5	2½	67	60	3	2	5	2½	67	58
7	5	10	4½	74	58	—	—	—	—	—	—
3	14	15	4	62	50	2	14	15	3½	67	56
(b) Kept in complete darkness.						(b) Normal light.					
2	2	Constant.	5½	48	71	4	—	—	6	55	69
7	5	"	8	53	91	8	—	—	6½	56	85
3	14	"	7	40	99	4	—	—	6	50	79

blood is reduced, the spleen is enlarged, and the vessels of the abdominal viscera are distended with multiple capillary hemorrhages.

The more detailed subsequent experiments confirmed and extended these results. We studied, particularly, in a qualitative way, the effect on the course of the trypanosome infection of various degrees of exposure of immersed and non-immersed animals to direct sunlight. The results are summarized in Table IV, *a*. The course of infection in controls kept under ordinary light conditions or in total darkness is shown in Table IV, *b*.

Although the results are only qualitative in character, they indicate

immersion in water or exposure to sunlight for short intervals may be referable to the same causes which are responsible for the production of malarial relapses on exposure to light or chilling. The relatively great resistance shown by animals kept under conditions of complete darkness accords with the results reported by Pearce and Van Allen (9) in experiments with a rabbit tumor.

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protein nitrogen was determined by the Folin and Wu method (3), and the chloride on the tungstic acid filtrate in the manner suggested by Gettler (4). Blood for the

TABLE I.

Viscosity of the Whole Blood and Plasma of the Normal Dog.

	Whole blood.	Plasma.
	<i>units</i>	<i>units</i>
Average (30 dogs).....	6.65	1.77
Highest.....	10.40	2.00
Lowest.....	4.50	1.50

TABLE II.

Obstruction of the Cardiac End of the Stomach.

Dog No.	Day after operation.	Blood (mg. per. 100 cc.).		Viscosity.		Hematocrit reading.
		Non-protein nitrogen.	Chlorides (as NaCl).	Whole blood (oxalated).	Plasma.	
		<i>mg.</i>	<i>mg.</i>	<i>units</i>	<i>units</i>	<i>per cent</i>
1	0	30	450	1.7	7.3	56
	1	43	440	2.1	11.0	55
	2	131	410	2.2	14.0	58
2	0	32	440	1.9	5.2	50
	1	115	360	2.3	10.0	53
3	0	26	450	1.7	9.6	—
	1	46	420	1.6	—	77
	2	48	390	1.9	9.6	64
	3	162	370	1.8	14.0	71
	4	238	360	2.1	21.0	64
4	0	31	480	1.5	6.0	55
	1	44	460	2.2	9.3	—
	2	121	450	2.6	10.8	43
5	0	32	460	1.7	7.4	54
	1	59	470	1.8	10.4	56
	2	145	410	2.0	14.4	68

determination was withdrawn from the jugular vein before operation and at 24 hour intervals thereafter until the end of the experiment. One small drop of a saturated solution of potassium oxalate was added for each 5 cc. of blood.

THE VISCOSITY OF THE BLOOD OF THE DOG AFTER OBSTRUCTION OF THE UPPER GASTRO-INTESTINAL TRACT.

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(Received for publication, May 27, 1926.)

Viscosity is a definite property of blood dependent upon the force with which the molecular constituents adhere to one another. Normally the viscosity of blood remains quite constant though influenced by a number of factors. The amount and type of protein in the plasma is a very important factor. Globulin is much more viscous than albumen. Knowing the viscosity of a given plasma it is possible to estimate its protein content. Viscosity of blood likewise varies with the corpuscle volume.

Estimations of viscosity of the blood have been little used in clinical medicine. Austrian (1) found no characteristic alterations in any disease. He concluded that the viscosity depends on the number of red corpuscles, the hemoglobin content, the gaseous richness, and to a lesser degree on the protein, fat, and salt content, but did not vary in direct proportion with any one factor. Bircher (2) has emphasized especially the predominant influence of the dissolved protein on viscosity.

As a part of a study of the physicochemical changes in the blood after experimental obstruction of the upper gastrointestinal tract we have made successive determinations of the viscosity of the plasma and of the whole blood.

Method.

Dogs were used for all experiments. All operations were done under ether anesthesia with aseptic technique. The obstruction of the cardiac end of the stomach and of the pylorus was made with a tape ligation. The jejunum was obstructed by severing the gut and inverting the cut ends.

The Hess viscosimeter was employed for the viscosity determinations. With this instrument the results are expressed in units of distilled water. The non-

EXPERIMENTAL OBSERVATIONS.

In Table I are summarized the viscosity determinations on thirty normal dogs. The average for the whole blood is 6.65 units and for the plasma 1.77 units. Quite wide variations were encountered.

TABLE IV.
Obstruction of the Jejunum.

Dog No.	Day after operation.	Blood (mg. per 100 cc.).		Viscosity.		Remarks.
		Non-protein nitrogen.	Chlorides (as NaCl).	Whole blood (oxalated).	Plasma.	
		mg.	mg.	units	units	
11	0	29	400	1.9	8.2	Died.
	1	44	320	2.2	8.5	
	3	88	280	1.7	8.0	
	4	53	280	1.8	6.2	
	7	75	230	1.7	6.3	
	8	78	200	1.7	5.2	
	10	102	190	1.7	4.2	
12	0	24	440	1.7	6.0	Killed.
	1	33	360	1.9	7.0	
	3	35	370	1.8	7.0	
	4	—	340	1.8	9.0	
	7	37	300	1.8	6.6	
	8	44	270	1.8	7.0	
	10	60	270	1.8	5.7	
	13	53	230	1.8	4.9	
13	0	27	470	1.7	4.8	720 cc. 1% NaCl.
	1	35	420	1.6	9.4	720 " 1" "
	3	31	410	1.8	5.4	720 " 2" "
	5	33	360	1.9	7.2	576 " 5" "
	7	40	360	1.7	6.2	Killed.
14	0	27	470	1.7	6.0	Given NaCl daily.
	1	35	430	1.7	8.0	
	2	—	350	1.7	8.0	
	5	65	350	1.7	6.3	
15	0	27	450	1.7	6.8	Given NaCl solution daily.
	1	37	430	1.7	7.5	
	2	—	460	1.7	7.5	
	5	27	440	1.7	8.0	

TABLE III.
Obstruction of the Pylorus.

Dog No.	Day after operation.	Blood (mg. per 100 cc.).		Viscosity.		Hematocrit reading.	Remarks.
		Non-protein nitrogen.	Chlorides (as NaCl).	Whole blood (oxalated).	Plasma.		
		mg.	mg.	units	units	per cent	
6	0	28	420	1.7	5.8	57	Died.
	1	102	320	1.9	11.0	63	
7	0	27	460	1.7	7.0	49	"
	2	59	360	2.1	15.0	—	
	3	141	360	1.5	14.0	69	
8	0	35	430	1.6	5.7	50	10 gm. NaCl by mouth. 10 " " " " 10 " " " " Recovered.
	2	32	330	1.8	14.0	—	
	3	30	330	1.7	9.2	72	
	4	33	310	2.2	10.2	63	
	5	38	290	2.0	10.8	56	
	6	42	320	2.3	7.7	62	
	7	60	360	2.1	9.0	—	
	9	79	320	2.0	7.6	50	
	10	68	350	2.1	11.0	45	
	11	39	470	1.8	4.8	42	
	12	23	500	1.7	4.8	40	
9	0	22	460	2.0	5.8	42	Obstruction released. Died.
	1	27	400	2.2	6.5	45	
	2	45	380	2.5	6.9	53	
	3	88	370	2.1	6.0	42	
10	0	25	450	1.9	6.0	50	Recovered.
	1	28	400	1.9	8.4	55	
	2	89	330	2.3	9.0	57	
	3	72	320	2.0	6.2	43	
	4	35	380	2.0	6.0	40	
	6	40	420	1.9	4.3	35	
	7	38	400	1.7	4.0	31	
	8	35	420	2.0	5.7	35	
	9	30	440	1.8	3.7	32	

With cardiac obstruction there is a rapid and marked rise in the viscosity of the whole blood and some increase in the viscosity of the plasma.

The changes after pyloric obstruction are similar to those observed after cardiac obstruction.

After obstruction of the upper jejunum only slight changes are observed.

The increase in viscosity parallels quite closely the degree of the toxemia. It is unaccompanied by any marked concentration of the blood.

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The determinations on five dogs with obstruction of the cardiac end of the stomach are shown in Table II. All animals showed a steady rise in viscosity which began before the rise in non-protein nitrogen was apparent. The increase was much more marked in whole blood than in plasma.

After obstruction of the pylorus (Table III) there is likewise a rapid and marked increase in viscosity of both whole blood and plasma. The increase parallels the event of the toxemia. One animal was treated with dry sodium chloride by mouth after the obstruction was released. The viscosity fell to a low point and the animal recovered.

After obstruction of the jejunum the results are variable (Table IV). There was usually little rise in viscosity. The change here did not run parallel with the rise in non-protein nitrogen or fall in chlorides.

DISCUSSION.

The marked change in viscosity of the blood after obstruction of the pylorus and cardiac end of the stomach is evidence of a physico-chemical change occurring in such conditions. The change in viscosity parallels quite closely the degree of toxemia. The toxemia after obstruction of the cardiac end of the stomach is more severe than with an obstruction at a lower level. Likewise the increase in viscosity is most marked here.

The explanation for the change is not definitely apparent. In cardiac obstruction there is a marked increase in fibrinogen which constitutes the major part of the globulin fraction of the plasma protein (5). Likewise there is a marked increase in the sedimentation rate of the erythrocytes which is dependent on agglutination of the cells. These two factors probably largely account for the changes observed.

Hematocrit readings were made daily on several animals with both pyloric and cardiac obstruction (Tables II and III). These show that there is no marked concentration of the blood, hence an increase in red cells cannot account for the change in viscosity.

SUMMARY AND CONCLUSIONS.

Estimations of the viscosity of the whole blood and plasma of the dog after experimental upper gastrointestinal tract obstruction are reported.

after obstruction. There is no constant change, however, even with the marked change in the non-protein nitrogen. With obstruction of the pylorus (Table III) there is also no characteristic change except

TABLE I.
Surface Tension of the Blood Serum of the Normal Dog.

	Initial.	After 2 hrs.	Time-drop.
	<i>dynes</i>	<i>dynes</i>	<i>dynes</i>
Average (20 dogs).....	64.0	61.5	3.5
Highest.....	67.5	66.2	4.7
Lowest.....	60.7	58.0	0.7

TABLE II.
Obstruction of the Cardiac End of the Stomach.

Dog No.	Day after operation.	Surface tension.			Blood.	
		Initial.	After 2 hrs.	Time-drop.	Non-protein nitrogen.	Chlorides (as NaCl).
		<i>dynes</i>	<i>dynes</i>	<i>dynes</i>	<i>mg.</i>	<i>mg.</i>
16	0	64.1	59.4	4.7	33	470
	1	62.7	56.7	6.0	40	480
	2	61.4	58.7	2.7	42	450
	3	63.5	59.4	4.1	150	420
17	0	62.7	61.4	1.3	30	510
	1	58.0	57.4	0.6	74	410
	2	60.0	57.4	2.6	185	490
18	0	64.8	61.4	3.4	37	480
	1	65.5	60.0	5.5	31	460
	2	62.7	59.4	3.3	74	430
4	0	64.8	61.4	3.4	31	480
	1	62.1	57.4	4.7	44	460
	2	65.5	59.4	6.1	121	450

an increase in the time-drop after the obstruction is made. Likewise with obstruction of the jejunum there is no typical change even with hypochloremia and a rise in the non-protein nitrogen.

SURFACE TENSION OF THE BLOOD SERUM OF THE DOG AFTER UPPER GASTROINTESTINAL TRACT OBSTRUCTION.

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While studying the physicochemical changes in the blood of the dog after obstruction of the upper intestinal tract we have determined the surface tension of the serum. Du Noüy has shown (1) that the surface tension of serum decreases as soon as it is exposed to the air. This phenomenon, designated "the time-drop," he considers an excellent index of the changes upon which surface tension depends.

Method.

Dogs were used for all the experiments. All operations were done under ether anesthesia with aseptic technique. The obstruction of the cardiac end of the stomach and of the pylorus was made with a tape ligation. The jejunum was obstructed by severing the gut and inverting the cut ends.

The du Noüy tensiometer (2) was used for all surface tension determinations. The non-protein nitrogen was determined by the Folin and Wu method (3), and the chloride on the tungstic acid filtrate in the manner suggested by Gettler (4). Blood for the surface tension and chemical determinations was withdrawn from the jugular vein before operation and at 24 hour intervals thereafter until the end of the experiment.

EXPERIMENTAL OBSERVATIONS.

In Table I are summarized the surface tension determinations on the blood serum of twenty normal dogs. The average initial reading was 64.0 dynes with a 2 hour time-drop of 3.5 dynes. The surface tension was found to vary little in normal dogs.

The surface tension readings on four dogs with obstruction of the cardiac end of the stomach are shown in Table II. The readings remain quite constant. The time-drop tends to increase 24 hours

SUMMARY AND CONCLUSIONS.

After obstruction of the upper gastrointestinal tract the surface tension of the blood serum of the dog shows no marked change. There is a tendency of the time-drop to increase 24 hours after operation. There is no characteristic course of the time-drop.

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TABLE III.
Obstruction of the Pylorus.

Dog No.	Day after operation.	Surface tension.			Blood.	
		Initial.	After 2 hrs.	Time-drop.	Non-protein nitrogen.	Chlorides (as NaCl).
		<i>dynes</i>	<i>dynes</i>	<i>dynes</i>	<i>mg.</i>	<i>mg.</i>
19	0	60.7	58.0	2.7	34	460
	1	65.5	59.0	6.5	46	320
	2	64.5	59.7	4.8	111	230
20	0	63.4	60.1	3.3	24	520
	1	66.8	58.2	8.6	28	480
	2	62.4	57.3	7.1	38	390
	3	62.4	57.4	5.0	126	320
7	0	64.2	61.4	3.7	29	460
	1	65.5	58.0	7.5	59	360
	2	61.7	58.3	3.4	141	360
21	0	64.1	60.7	3.4	31	500
	1	66.8	60.0	6.8	35	450

TABLE IV.
Obstruction of the Jejunum.

Dog No.	Day after operation.	Surface tension.			Blood.	
		Initial.	After 2 hrs.	Time-drop.	Non-protein nitrogen.	Chlorides (as NaCl).
		<i>dynes</i>	<i>dynes</i>	<i>dynes</i>	<i>mg.</i>	<i>mg.</i>
22	0	63.5	60.0	3.5	24	470
	1	64.8	60.0	4.8	25	440
	2	63.5	60.0	3.5	88	420
23	0	63.5	59.4	4.1	54	470
	1	61.4	57.4	4.0	33	440
	2	61.4	56.7	4.7	34	430
24	0	61.4	62.1	0.7	26	460
	1	61.4	58.0	3.4	41	410
	2	63.5	61.4	2.1	35	330
	3	60.7	59.0	1.7	50	360
	4	65.5	60.7	4.8	34	400
	5	66.2	64.4	1.8	35	330
25	0	64.8	65.5	0.7	38	460
	1	65.5	59.4	6.1	39	400
	2	64.1	62.4	1.7	44	370
	3	66.8	64.8	2.0	60	330
	4	63.5	60.0	3.5	33	320

Method.

All experiments were made on dogs. No food was given after operation or for 24 hours before operation. All operations were done under ether anesthesia with aseptic technique. Blood for examination was obtained from the jugular

TABLE I.

Sedimentation Time of the Erythrocytes of the Normal Dog.

	Time required to sediment.			
	6 mm.	12 mm.	18 mm.	24 mm.
	min.	min.	min.	min.
Average (20 dogs).....	653	1045+	1068+	1084+
Longest.....	1440+	1440+	1440+	1440+
Shortest.....	12	22	75	60

TABLE II.

Obstruction of the Cardiac End of the Stomach.

Dog No.	Day after operation.	Time required to sediment.				Blood.	
		6 mm.	12 mm.	18 mm.	24 mm.	Non-protein nitrogen.	Chlorides (as NaCl).
		min.	min.	min.	min.	mg.	mg.
16	0	1260	—	—	—	33	470
	1	105	255	1320	—	40	480
	2	30	75	150	1440	42	450
	3	—	45	55	240	150	420
18	0	330	1440	—	—	37	480
	1	35	57	120	1440	31	460
	2	25	37	55	255	74	430
4	0	1440	—	—	—	31	480
	1	90	270	—	—	44	460
	2	40	50	70	—	121	450
3	0	1440	—	—	—	26	450
	2	—	85	330	—	48	390
	4	60	—	—	—	238	360

vein before operation and at 24 hour intervals thereafter until the experiment was ended by the death or recovery of the animal. The obstruction of the pylorus and cardiac end of the stomach was made with a tape ligature tightly drawn.

THE SEDIMENTATION RATE OF THE ERYTHROCYTES IN EXPERIMENTAL OBSTRUCTION OF THE GASTROINTESTINAL TRACT.

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The time required for the red cells to separate from the plasma of citrated blood varies widely. Normally they remain suspended over a long period, since all erythrocytes carry a negative charge and thus repel each other. The maintenance of the electric charge is the principal stabilizing factor. Under abnormal conditions the cells may lose the charge, agglutinate, and precipitate rapidly.

Fachreus (1) has shown that the change in sedimentation rate is a physico-chemical phenomenon dependent upon a variation in the properties of the plasma. Cells which in one plasma separate rapidly, when placed in the plasma of a normal individual show a normal sedimentation time; cells from a normal blood when added to the plasma of a blood with a rapid sedimentation rate, separate out quickly. There is a qualitative change in the protein; an increase in the amount alone has no effect. Faehreus found a relative increase in the serum globulin, principally in the fibrinogen factor, of the blood when the sedimentation rate is increased. Globulin has a high agglutinating power; albumen agglutinates to only a slight extent. The agglutinative capacity of a protein seems related to its general colloidal state; consequently the suspension stability of the cells is dependent, in the last analysis, upon a change in the colloidal state of the plasma.

Tests of the sedimentation rate have been utilized in clinical medicine, especially in obstetrics and gynecology (2-4). In numerous clinical conditions the cells settle out more rapidly than normal. Pregnancy is associated with a marked change in rate. In general it has been found that in any condition in which there is an increased protein destruction, the sedimentation rate is more rapid than normal.

We have studied the suspension stability in a series of dogs in which an obstruction in the upper gastrointestinal tract had been experimentally produced.

The time required to separate to deeper levels was proportionately longer.

In Table II are shown the sedimentation rates in four dogs with obstruction of the cardiac end of the stomach. In each animal there is a marked increase in the rapidity of settling after operation. This change does not parallel the level of the chlorides and non-protein nitrogen. Much the same results are observed in obstruction of the

TABLE IV.
Obstruction of the Jejunum.

Dog No.	Day after operation.	Time required to sediment.				Blood.	
		6 mm.	12 mm.	18 mm.	24 mm.	Non-protein nitrogen.	Chlorides (as NaCl).
		<i>min.</i>	<i>min.</i>	<i>min.</i>	<i>min.</i>	<i>mg.</i>	<i>mg.</i>
23	0	310	1500	1740	—	33	470
	1	250	490	—	—	33	440
	2	30	80	340	—	34	430
28	0	1380	—	—	—	35	480
	2	75	110	165	1440	30	440
	4	—	20	30	80	51	400
	6	30	42	50	65	119	290
22	0	75	180	—	—	24	470
	1	90	165	255	420	25	440
	2	—	65	115	—	88	420
24	0	1620	—	—	—	26	460
	1	120	1380	—	—	41	410
	2	120	320	1500	—	35	330
	3	23	50	—	150	50	360

pylorus (Table III). After obstruction of the jejunum there is also a striking change in the sedimentation rate which does not parallel variations observed in the chemical study of the blood.

DISCUSSION.

The striking variations in the suspension stability of the blood observed in these experiments emphasize the changes taking place in the physicochemical state of the blood after obstruction of the upper

The jejunum was obstructed by severing the gut about 24 inches below the pylorus and turning in the cut ends.

The non-protein nitrogen was determined by the method of Folin and Wu (5); the chlorides were estimated on the tungstic acid filtrate in the manner suggested by Gettler (6). In determining the sedimentation rate Lintzenmeier tubes were employed as described by Friedlander (2).

TABLE III.
Obstruction of the Pylorus.

Dog No.	Day after operation.	Time required to sediment.				Blood.	
		6 mm.	12 mm.	18 mm.	24 mm.	Non-protein nitrogen.	Chlorides (as NaCl).
		<i>min.</i>	<i>min.</i>	<i>min.</i>	<i>min.</i>	<i>mg.</i>	<i>mg.</i>
26	0	1260	—	—	—	31	470
	2	—	80	150	1440	40	360
	4	45	—	105	255	67	350
	6	10	30	42	115	55	340
	8	—	20	30	50	70	340
27	0	48	335	—	—	12	490
	2	23	27	35	100	79	320
	4	14	17	25	120	111	330
	6	10	14	20	45	103	390
	8	—	12	17	22	108	470
1	0	1440	—	—	—	29	490
	1	60	—	160	—	27	450
	2	—	—	—	—	61	380
	3	17	27	39	107	77	340
8	0	120	1440	—	—	35	430
	2	75	210	240	—	30	300
	4	20	—	45	75	38	290
	6	15	25	45	90	60	360
	8	15	27	40	70	68	320
	10	15	21	30	45	26	500

EXPERIMENTAL OBSERVATIONS.

The sedimentation rate of the normal dog was found to vary widely. In Table I is shown the average for twenty dogs. The average time required to sediment even 6 mm. was approximately 11 hours. The longest time observed was over 24 hours and the shortest 12 minutes.

gastrointestinal tract. The sedimentation rate is affected much more quickly than is the level of the chlorides and non-protein nitrogen.

The sedimentation rate follows closely the degree of agglutination of the red cells. This may be well demonstrated by placing a drop of the citrated blood on one end of a clean glass slide and allowing it to run down to the other end. With normal blood this is uniformly smooth. If the sedimentation time is rapid the blood film has a granular appearance due to the agglutination of the erythrocytes.

SUMMARY AND CONCLUSIONS.

The results of the study of the suspension stability of the blood after upper gastrointestinal tract obstruction are reported.

After obstruction of the cardiac end of the stomach, of the pylorus, and of the jejunum the red cells separate from the plasma much more rapidly than normal.

The variation in sedimentation time is due to a variation in the physicochemical status of the blood.

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EXPERIMENTAL OBSERVATIONS.

The chloride content (as NaCl) of the liver and muscle of twenty normal dogs is shown in Table I. The average for the liver is 1.44 gm. per kilo and for muscle 1.06 gm. per kilo of tissue. In Table II

TABLE I.
Tissue Chlorides of the Normal Dog.

	Chlorides as NaCl (gm. per kilo).	
	Liver.	Muscle.
	gm.	gm.
Average (20 dogs).....	1.44	1.06
Highest.....	2.10	1.67
Lowest.....	1.05	0.76

TABLE II.
Obstruction of the Pylorus.

Dog No.	Day after operation.	Blood (mg. per 100 cc.).		Tissue chlorides (as NaCl in gm. per kilo).	
		Non-protein nitrogen.	Chlorides (as NaCl).	Liver.	Muscle.
		mg.	mg.	gm.	gm.
20	0	24	520	2.10	1.16
	3	126	320	0.98	0.85
21	0	31	500	1.40	1.09
	3	35	450	1.26	0.90
1	0	29	490	1.50	0.94
	5	181	260	0.77	0.70
7	0	27	460	1.93	0.84
	4	141	360	1.70	1.15

are summarized the determinations on four dogs with pyloric obstruction. All animals showed the characteristic fall in chlorides and rise in non-protein nitrogen of the blood. The chloride content of the liver decreased in each animal. This was very marked in the two

THE CHLORIDE CONTENT OF THE TISSUES OF THE DOG AFTER EXPERIMENTAL GASTROINTESTINAL TRACT OBSTRUCTION.

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The chloride content of the blood of the dog shows a progressive decrease after experimental obstruction of the pylorus (1) or upper intestine (2). A similar, though less marked, change takes place if the cardiac end of the stomach is ligated (3). In pyloric and intestinal obstruction some chloride is lost through vomiting. Vomiting obviously plays no part in the chloride decrease observed in cardiac obstruction. In other clinical conditions, notably lobar pneumonia, a decrease in the chloride content in the entire absence of vomiting is a characteristic finding (4). A series of determinations of the chloride content of the blood by Van Slyke's method (5) have shown that the chloride is not so fixed that it cannot be determined by the usual chloride determination on the tungstic acid filtrate (6). We have recently made for comparison with the blood chloride a series of chloride determinations on the liver and muscle of dogs in which an obstruction of the upper gastrointestinal tract had been experimentally produced.

Method.

All chloride determinations on liver and muscle were made by Van Slyke's method (5). Specimens of tissue were removed at operation when the obstruction was produced, and again at autopsy immediately after the death of the animal. The non-protein nitrogen of the blood was determined by the Folin and Wu method (7), and the chloride on the tungstic acid filtrate in the manner suggested by Gettler (8).

the tissue chlorides are similar to those observed after pyloric obstruction (Table III). The chloride content of the liver decreased in all animals. In one of them the chloride of the muscle was increased, in one no change was observed, and in one there was a decrease. The four animals with cardiac obstruction showed little change. The chloride content of the liver of each animal showed a slight rise. In three animals there was also a slight rise in the chloride of the muscle.

SUMMARY AND CONCLUSIONS.

Comparative observations are reported on the chloride content of the blood, liver, and muscle of the dog after obstruction of the upper gastrointestinal tract.

After obstruction of the pylorus there is a marked fall in the chloride content of the liver, and a slight fall in the muscle.

Similar changes are observed after obstruction of the jejunum.

With obstruction of the cardiac end of the stomach no change was observed.

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TABLE III.
Obstruction of the Jejunum.

Dog No.	Day after operation.	Blood (mg. per 100 cc.).		Tissue chlorides (as NaCl in gm. per kilo).	
		Non-protein nitrogen.	Chlorides (as NaCl).	Liver.	Muscle.
		mg.	mg.	gm.	gm.
24	0	26	460	1.43	1.18
	8	69	350	1.04	1.71
29	0	30	520	1.52	1.14
	1	32	410	1.31	0.76
30	0	29	480	1.70	0.85
	7	50	350	1.10	0.84
12	13	53	230	0.60	0.47

TABLE IV.
Obstruction of the Cardiac End of the Stomach.

Dog No.	Day after operation.	Blood (mg. per 100 cc.).		Tissue chlorides (as NaCl in gm. per kilo).	
		Non-protein nitrogen.	Chlorides (as NaCl).	Liver.	Muscle.
		mg.	mg.	gm.	gm.
18	0	37	480	1.16	1.08
	3	74	430	1.24	1.19
4	0	31	480	1.44	1.19
	2	121	450	1.55	1.20
31	0	29	500	1.42	1.67
	2	55	440	1.54	2.20
3	0	26	460	1.05	0.79
	6	238	360	1.20	0.67

dogs having a marked drop in blood chlorides. The chloride content of the muscle decreased in three of the four animals, but much less than that in the liver. After obstruction of the jejunum the changes in

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JOHN H. NORTHROP

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group strains. However, when a serum is absorbed with an heterologous strain of a group, and the absorbing dose is equivalent to the dose of the homologous strain necessary for the absorption of the homologous agglutinin, all heterologous agglutinin is removed. Such absorption not only removes the agglutinin for heterologous strains of the group represented by the serum, but the agglutinin for strains of the other group as well. It is only by excessive and repeated doses of the absorbing antigen under conditions where the agglutinin is liable to deteriorate from long continued heating that the homologous agglutinin can be absorbed by heterologous group strains. Apparently the antigenic characteristic of the individual strain is paramount while the group characteristics are of secondary importance in the bacterial structure.

The most satisfactory explanation for the antigenic relationships apparently existing between the strains of the erysipelas and scarlatinal groups, is that the antigen in each strain is a mosaic of antigenic fractions which occur in variable amounts in different strains. Group specificity would then depend on the presence of characteristic fractions some of which might occur in certain strains of the other group. The predominating fraction would give the strain specificity, a characteristic antigen or group of antigens would give group specificity, and the occurrence of one or more of these fractions in both groups would explain the apparently existing relationship between scarlatinal and erysipelas strains.

We have previously studied the absorption of agglutinin by several methods. In 1924 we studied the scarlatinal group (2). We attempted to absorb the agglutinin for the homologous strain from its corresponding serum with heterologous scarlatinal strains. We were able to accomplish our purpose but with certain reservations as far as strains and sera were concerned. The following quotation is taken from the published article.

"We have found that certain combinations of sera and strains are necessary for the successful absorption of agglutinin. These combinations can usually be picked out by the speed of the reaction and the degree of agglutination. Strains will absorb the agglutinin from sera which are fresh if the agglutination is complete within 15 minutes at 55°C.

By selecting sera and strains which have reacted vigorously we have been able to obtain a series of absorption tests in which every strain is represented."

STUDIES ON THE BIOLOGY OF STREPTOCOCCUS.

VI. BIOLOGY OF HEMOLYTIC STREPTOCOCCUS: ANTIGENIC RELATIONSHIPS BETWEEN STRAINS OF THE SCARLATINAL AND ERYSIPELAS GROUPS.

BY FRANKLIN A. STEVENS, M.D., AND A. R. DOCHEZ, M.D.

(*From the Medical Clinic of Presbyterian Hospital, Columbia University, New York.*)

(Received for publication, June 10, 1926.)

In a recent study (1) of agglutination and absorption of agglutinin with strains of hemolytic streptococcus from scarlet fever and erysipelas, we found these two groups to be related antigenically. This relationship was peculiar in that the strains within the groups were not identical, yet possessed of common antigenic properties. These common antigenic properties were apparently not entirely group-specific, since sera of one group would agglutinate certain strains of the other group of streptococci. When several erysipelas strains and their corresponding sera were employed in a comprehensive series of agglutination reactions, in which each of the strains was tested for agglutination with each of the sera, few negative reactions were found. Except with strains which were apparently physically incapable of agglutination, the reactions within the group were consistently positive with each strain and serum. When scarlatinal strains were agglutinated with these erysipelas immune sera, by testing each of the scarlatinal strains for agglutination with each of the sera in a manner similar to the tests carried out with the erysipelas strains, a much lower percentage of positive reactions was obtained. None of the strains and none of the sera showed consistently positive reactions. A strain might agglutinate in one or several of the erysipelas sera. A serum might agglutinate one or more of the scarlatinal strains. The antigens of the two groups were apparently related.

Absorption of agglutinin has shown that the strains within the groups are not identical, since it is not usually possible to absorb the agglutinin for the homologous strain from serum with heterologous

The results of these absorptions and agglutinations are arranged in Tables I to III. Each table represents the work with a single serum. The absorbing strain is indicated at the head of each broad column and the decimal part of the unit absorbing dose employed is found at the left margin. The numerals at the intersecting points show the degree of agglutination with the absorbed serum and the various scarlatinal and erysipelas strains. The agglutinations were done in a series of six dilutions, 1:40, 1:80, 1:160, 1:320, 1:640,

TABLE I.

Fractional Agglutinin Absorption: Erysipelas Serum I Absorbed with Erysipelas Strains I, II, and III.

Absorbing dose	Absorbed by Strain E I								Absorbed by Strain E II								Absorbed by Strain E III							
	E I	E II	E III	E VII	S I	S VIII	S IX	S XV	E I	E II	E III	E VII	S I	S VIII	S IX	S XV	E I	E II	E III	E VII	S I	S VIII	S IX	S XV
1.5	—	—	—	—	—	—	—	—	5	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
1.0*	3	—	—	—	—	1	—	—	5	—	—	—	—	—	—	—	4	—	—	—	—	—	—	—
0.5	6	—	—	—	—	2	—	—	5	—	—	—	—	—	—	—	4	—	—	1	—	—	—	—
0.45	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
0.4	6	—	—	3	—	3	—	1	5	—	—	—	1	1	—	—	6	—	—	3	—	1	—	—
0.35	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
0.3	6	—	2	2	1	3	—	2	6	—	1	—	2	1	—	—	—	—	—	—	—	—	—	—
0.25	6	—	1	3	1	3	—	3	6	—	1	1	3	4	—	1	5	—	—	4	3	3	—	4
0.2	6	2	2	3	—	4	—	2	6	—	4	3	3	4	—	1	5	—	1	6	4	4	1	4
0.15	6	2	2	5	4	5	1	5	6	—	5	3	3	4	—	3	5	1	1	6	5	5	3	5
0.1	6	2	4	6	4	4	3	4	6	1	6	5	5	4	1	4	6	1	2	6	5	5	4	6
0.05	6	4	4	6	6	6	2	5	6	2	6	6	6	6	3	4	5	1	2	6	5	6	4	6
0.0	6	5	6	6	6	6	3	6	6	4	6	5	6	6	3	6	—	—	—	—	—	—	—	—

* Absorbing doses are in fractions and multiples of this unit.

and 1:1280. The numerals 1, 2, 3, 4, 5, and 6 indicate respectively the last dilution in which agglutination occurred. The scarlatinal and erysipelas strains have been indicated by the appropriate letters S and E.

DISCUSSION AND SUMMARY.

The interpretation of the results in these experiments is difficult on account of the variations in the physical capacity of different strains of hemolytic streptococcus to agglutinate and absorb agglu-

These reactions were carried out with doses of antigen which we now consider excessive for absorption work. In an immediately previous article published in 1926 (1) the dose of antigen employed in absorbing the sera was uniform. It was a dose slightly larger than that required when the homologous agglutinin was absorbed from a serum for the corresponding strain. With this dose it was seldom possible to absorb the homologous agglutinin with heterologous strains of the group. While these absorption tests demonstrated what all the strains of a group were not identical they did not indicate how closely the individual strains might resemble each other. A series of absorption reactions has been carried out in which the sera were absorbed with doses of antigen uniformly and gradually increased from the unabsorbed serum ("zero" dosage) to the dose necessary for the absorption of the homologous strain. Both scarlatinal and erysipelas strains have been included so as to determine the relationship between the strains in the erysipelas group as well as their relationship to those strains of the scarlatinal group which agglutinate in erysipelas immune sera.

Experimental Methods.

Three erysipelas immune sera and a number of erysipelas and scarlatinal strains were selected for the agglutination and absorption tests. The erysipelas strains were chosen without discrimination but scarlatinal strains were picked which agglutinated with the three sera. Since agglutination of each of these scarlatinal strains did not occur with all of the sera, the same strains could not be employed throughout the experiments. The technique of immunization and agglutination have been fully described previously (1, 2). The method of absorption also has been described with the exception of the technique for obtaining uniform absorptive doses and of the manner in which the fractional doses were estimated.

The physical bulk of streptococci necessary for the subminimal absorption of agglutinin was determined for each serum and the homologous strain. The streptococci were grown in broth and centrifuged under uniform conditions in especially constructed quantitative centrifuge tubes. The quantities necessary with each of the sera and the homologous strain were averaged, and this average was accepted as the unit absorptive dose of bacteria for a definite volume of serum of 1:20 dilution. Each of the three erysipelas sera was then absorbed with fractional and multiple doses of this unit. Each of the three sera was absorbed in turn by all three strains represented by the immune sera. After each absorption the sera were tested for residual agglutinin with erysipelas and scarlatinal strains.

tion by heterologous strains failed to absorb the agglutinin for the strain homologous with the serum. This strain individuality is again apparent in Tables I to III. Doses of heterologous strains equivalent to the unit absorptive dose which has already been defined, fail to remove the agglutinin for the homologous strain. This dose is sufficient however, to absorb the agglutinin for practically all heterologous strains. It appears from this observation that the strain specificity dominates the group specificity.

TABLE III.

Fractional Agglutinin Absorption: Erysipelas Serum III Absorbed with Erysipelas Strains I, II, and III.

Absorb- ing dose	Absorbed by Strain E I										Absorbed by Strain E II										Absorbed by Strain E III									
	E I	E III	E VI	E VII	E IX	S I	S III	S VI	S VIII	S XV	E I	E III	E VI	E VII	E IX	S I	S III	S VI	S VIII	S XV	E I	E III	E VI	E IX	S I	S III	S VI	S VIII	S XV	
1.5	—	3	—	—	—	—	—	—	—	—	—	2	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	
1.0*	—	2	—	—	—	—	—	—	—	—	—	2	—	—	—	—	—	1	—	—	—	—	—	—	—	—	—	—	—	
0.5	—	3	—	—	—	—	—	—	—	—	—	2	—	—	—	—	—	1	—	—	—	—	—	—	—	—	—	—	—	
0.45	—	3	—	—	—	—	—	—	—	—	—	4	1	—	—	1	—	1	1	—	—	1	—	—	—	—	—	—	—	
0.4	—	3	—	—	—	—	—	—	—	—	—	4	1	—	—	1	—	1	1	—	—	1	—	—	—	—	1	—	—	
0.35	—	3	—	—	1	—	—	2	—	—	1	2	3	—	—	4	—	3	2	—	1	4	—	—	—	—	1	—	—	
0.3	—	3	—	—	—	—	—	—	—	—	—	3	3	1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	
0.25	1	4	3	—	3	1	—	2	—	—	3	3	1	—	—	2	1	1	4	2	1	3	—	—	—	—	2	1	—	
0.2	1	5	3	—	3	—	—	4	1	2	4	5	1	—	—	2	2	3	4	2	1	4	3	1	1	—	5	3	1	
0.15	4	5	4	—	5	3	1	3	4	1	5	4	2	—	1	4	1	5	3	4	4	5	3	1	1	1	4	4	3	
0.1	5	4	4	1	6	3	2	5	5	3	5	6	2	—	4	5	4	4	5	4	4	5	—	—	3	3	4	4	4	
0.05	5	5	6	3	6	4	4	5	6	5	4	5	4	—	4	6	4	5	6	6	5	6	—	—	4	3	5	4	4	
0.0	6	6	6	4	6	5	3	6	6	5	6	6	4	—	5	6	5	5	5	6	—	—	—	—	—	—	—	—	—	

* Absorbing doses are in fractions and multiples of this unit.

Between the unit absorptive dose and a dose which is approximately 0.2 to 0.3 of this unit, is a zone of great variation in absorption and agglutination. Below this zone absorption is complete for few strains. The differences in titer of various strains and absorbed sera in the zone where variations occur are very definite. For example in Table II, Strain E IX agglutinates well in Serum II absorbed with Strains E I and E III but poorly in serum absorbed with Strain E II. Numerous similar examples may be found. In a few instances heterologous strains agglutinate nearly as well as the homologous strains in these

tinin. Even when the antigens for agglutination and absorption are standardized and sera are carefully titrated variations in agglutination occur with different strains and sera. Variations may occur because of differences in the titer of the immune sera employed. In a large series of reactions with the same sera and strains, errors in interpretation depending on these factors can be eliminated because strains which agglutinate poorly will consistently give weak reaction with all sera, and weak sera will agglutinate all strains poorly. After such varia-

TABLE II.

Fractional Agglutinin Absorption: Erysipelas Serum II Absorbed By Strains E I, II, and III.

Absorb- ing dose	Absorbed by Strain E I										Absorbed by Strain E II								Absorbed by Strain E III										
	E I	E II	E VI	E VII	E IX	S I	S VII	S IX	S XI	S XV	E I	E II	E VI	E VII	E IX	S I	S IV	S VII	S IX	E I	E II	E VI	E VII	E IX	S I	S VII	S IX	S XI	S XV
1.5	4																			5									
1.0*	6										1									6									
0.5	6										4									6				2					
0.45																				6				3					
0.4	4								1		5									6				3					
0.35									3	2	6									4				3					
0.3	6	1							5	4		6	1							4				1	3		1		
0.25	6	5						1	6	6		6	3							6	6			1	3		3		1
0.2	6	6		1	6			2	6	5	1	6	3							6	6			3	4		1	4	1
0.15	6	6	1	3	3	1	6	6	6	6	2	6	3			5				6	6		2	4	4		1	5	3
0.1	6	6	2	3	6	3	6	6	6	4	5	4	2		5					4	5	6	3	1	5	6	1	3	4
0.05	6	6	3	4	6	4	6	6	6	3	6	6	6	4	6	2	2	4	6	6	6	3	4	6	6	3	6	6	4
0.0	6	6	4	5	6	4	6	6	5	4	6	6	5	6	6	4	4	6	6	6	6	5	6	6	3	6	6	6	3

*Absorbing doses are in fractions and multiples of this unit.

tions in agglutination are eliminated, if a strain varies grossly in agglutination with different sera, or the titer of different sera varies with the same strain as in Tables I to III, variations which occur must represent antigenic dissimilarities.

We have previously commented on the dissimilarity of the individual strains in the scarlatinal and erysipelas groups. Although the strains within each of the groups are related they are seldom identical. This individuality of the strain was indicated by the fact that absorp-

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absorbed sera. Such strains probably resemble the homologous strains closely. These resemblances are not confined to the erysipelas strains but occur among the scarlet strains which agglutinate in erysipelas sera. Apparently it is impossible to distinguish such scarlatinal strains from erysipelas strains by absorption of agglutinin.

The relationship shown by these absorption tests is additional evidence in favor of the mosaic nature of the antigen. Strains of streptococcus of the erysipelas groups are apparently composed of several agglutinogenic fractions. If the antigen of a strain is made up of the fractions A, B, C, D, and E, serum for that strain would contain A, B, C, D, and E agglutinins. If this serum were absorbed with the homologous strain or an identical heterologous strain the agglutinin would be completely absorbed. On the other hand a qualitative or quantitative difference between the two strains would be apparent in the absorption and agglutination reactions. Such differences are evident in Tables I to III. This serum would agglutinate any strain containing any one or several of the fractions, but absorption of the serum by such a strain would leave fractions of the agglutinin in the serum. This absorbed serum would agglutinate the homologous strain. Heterologous strains would agglutinate if fractions remained in the serum corresponding to their antigenic components. Hence the reactions in the absorbed serum would depend on the qualitative and quantitative relationships between the component fractions in the serum, the absorbing strain, and the strains agglutinated.

This conception of multiple antigens is not new in bacteriology. Durham (3) explained the reactions in the colon-typhoid group of bacilli on this basis. More recently Durand and Sédallian (4), and Andrewes, Derick, and Swift (5) have expressed the opinion that the agglutination reactions with hemolytic streptococcus can only be accounted for in this way. In many respects the reactions observed with the colon-typhoid group of Gram-negative bacilli resemble those experienced with the erysipelas and scarlatinal groups of hemolytic streptococcus. Apparently we have exhausted the possibilities of studying these groups further by agglutination and absorption. Our knowledge regarding the specificity and relationship of the antigenic fractions must come from the study of fractions isolated and refined by chemical methods.

shine together with the rate, extent, and persistence of change in the curve of sunshine over a given period could be related to the character of the experimental diseases. Furthermore, when the curve of sunshine was compared with the organ weights of a sufficiently large number of normal rabbits examined during the time of our experiment, it was found that the fluctuations of the curve corresponded with variations in the mass relationships of practically all the organs, and perhaps most strikingly so in the case of certain of the ductless glands and representative lymph nodes. Since our experiments were in each instance carried out with rabbits kept in rooms which received practically all sunlight only as filtered through ordinary window glass, the effects observed cannot be attributed wholly to the short or so called therapeutic ultra-violet rays.

The observations led to the conception of a relationship between the nature or character of light, on the one hand, and the physical state and functional activities of the animal organism, on the other. In order to test this conception experimentally, an investigation was undertaken in which conditions of light could be controlled. Since the significant features of the factor of sunlight appeared to be its total amount together with the change in actual hours of sunshine, our initial experiments were carried out with a constant illumination of the maximum intensity that was practicable with a simple equipment. Its effect was studied, first, upon the normal rabbit with especial reference to organ weights, and second, upon the course of the malignant tumor and of experimental syphilis. A summary of the first experiment dealing with the results of tumor inoculation has already appeared (1), and those in which *T. pallidum* was employed will be published shortly.

The results of the experiment with normal rabbits are reported in the present series of papers. The methods employed, the clinical observations, and the postmortem findings are described in Paper I. In Paper II, the actual organ weights and the weights of organs per kilo of net body weight are presented in tabular form. An analysis of these statistical data, including a consideration of their reliability as an index of existing conditions, is discussed in Paper III.

EFFECTS OF LIGHT ON NORMAL RABBITS, WITH ESPECIAL REFERENCE TO THE ORGANIC REACTION.

I. CLINICAL AND POSTMORTEM OBSERVATIONS.

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For several years a study of the animal organism in these laboratories has included an investigation of the mass relationships of various organs with especial reference to the subjects of variation, the effect of environmental influences, and the relation of these conditions to phenomena of animal resistance as indicated by the occurrence and general course of disease.

A striking feature both of experimental syphilis and of a transplantable neoplasm of the rabbit is the variability of the disease manifestations, and observations over a period of several years have led us to the opinion that a relationship exists between the season of the year and the general character of the disease. It was further found that the weights of many organs not directly involved by the disease process were affected and that these alterations could be correlated with the character and clinical course of the disease. In addition, variations of a rhythmic character which appeared to have a seasonal significance could be demonstrated in the organ weights of normal rabbits, and the organs in which these changes were especially marked were those in which the more pronounced weight alterations were observed in association with syphilis and the malignant tumor.

The influence of the seasonal factor has been interpreted as affecting animal economy, while susceptibility or resistance to disease has been considered as a function of the animal organism. Among the several factors contributing to weather conditions during the time of our experiments, sunshine appeared to be most closely correlated with variations in disease manifestations. That is, the actual hours of sun-

rabbits designated as "outside" controls were killed and examined at the same time.² These animals had been in the laboratory for only a few days and represent a stock which had been living under unknown but presumably different conditions from those of the control rabbits in the experiment.

Upon the day the rabbits were killed the feeding was omitted; they were weighed and then killed by air embolism, a method quicker and less disturbing than etherization. Each animal was at once bled from the inferior vena cava and the autopsy performed immediately. The details of our method of post-mortem examination are elsewhere described (2). The organs weighed were: the gastrointestinal mass, the heart, the liver, the kidneys, the spleen, the brain, the thyroid, the parathyroids, the hypophysis, the suprarenals, the pineal, the thymus, the testicles, the posterior axillary and popliteal lymph nodes, the deep cervical lymph nodes, and the main mass of mesenteric nodes. In order to avoid drying after removal from the body, the small organs were placed in Petri dishes and kept moist with physiological salt solution. Before weighing the excess fluid was removed by absorption on filter paper.

The records kept included the source of each rabbit, the date of receipt, an age grouping, a breed or type classification, a weight chart, and a record of the general physical condition of the animal during the period of observation.

The statistical analysis of the organ weights has included both actual and relative weights, that is, the weight of organs per kilo of net body weight.

No animal has been omitted because of any abnormality detected either during life or at autopsy, and the figures, as given, include all data irrespective of whether an organ was normal or pathological. Certain groups contain one or more goiters and in these instances additional figures for the thyroid are given from which these weights have been omitted. Similar supplementary figures are given for the deep cervical lymph nodes in those cases in which very large nodes were associated with a purulent infection of the middle ear or cranial sinuses.

RESULTS.

The clinical observations on the 150 rabbits comprising the experiment were made over a period of 6 to 16 weeks, reckoning from the time the animals were first brought to the laboratory; while the dura-

1925. These observations are not included in the present paper because of the introduction of a number of factors not uniform with those reported. Much of the data from this additional experiment, however, is in general conformity with that of the first.

² No outside control rabbits are available for the last group of experimental animals. We have, however, included the results obtained from 20 normal rabbits killed and examined on March 12, midway between the time when the fourth and fifth experimental groups were examined. These animals had been caged indoors for 1 to 4 weeks.

Methods and Materials.

The rooms in which this experiment was carried out were three in number. One, the so called "light" room, measuring $19 \times 9.5 \times 10.25$ feet or including 1850 cubic feet, was kept constantly and solely illuminated by 1000 watt Mazda lamps and Cooper Hewitt 50 inch low pressure mercury arcs, Type P, in crown glass, arranged in three superimposed horizontal rows on the long axis of the room. Each row contained four Mazda lamps and one mercury arc. The animal cages were placed in racks along the sides of the room parallel with and equidistant from the source of lighting. The intensity of the light reaching the cages averaged 425 foot candles; these determinations were made by the Electrical Testing Laboratories to whom our appreciative thanks are due. The spectrogram supplied by the Cooper Hewitt Company shows that the crown glass of the mercury arc does not transmit light below wave-lengths of 3022-28 Ångström units, while a spectrogram of the Mazda lamps which was kindly made for us by Dr. Frederick L. Gates shows that their light is cut off at about 3100 Ångström units. For the purpose of the present experiment there was no need to analyze further the nature or quality of the light.

In the "dark" room all light was constantly excluded except for the time, approximately 1 hour per day, when the animals were fed or examined. On these occasions a single 30 watt Mazda lamp was used. Satisfactory ventilation and temperature regulations of both the light and dark rooms were obtained by a system of fans and ventilating vents.

The "control" room was an unaltered animal room; it has a southern exposure and is lighted by two large windows. During the time of the experiment practically all the sunlight in this room diffused through the ordinary lead glass of these windows.

The experiment was conducted as follows: 150 male rabbits were assembled from November 14 to December 15, 1924, separately caged, and kept in ordinary animal rooms. The rabbits were chosen from carefully selected stocks of well nourished animals free from any external manifestations of disease. They were obtained chiefly from breeders or dealers in eastern Pennsylvania and included all the ordinary breeds of rabbits that are commonly used in our laboratory. The various breeds were proportionately distributed in the major divisions and sub-groups of the experiment. The exact age of the animals was unknown but the great majority of them were between 8 months and 1 year old. The diet of all was the same throughout the experiment and consisted of hay, oats, and fresh cabbage or carrots.

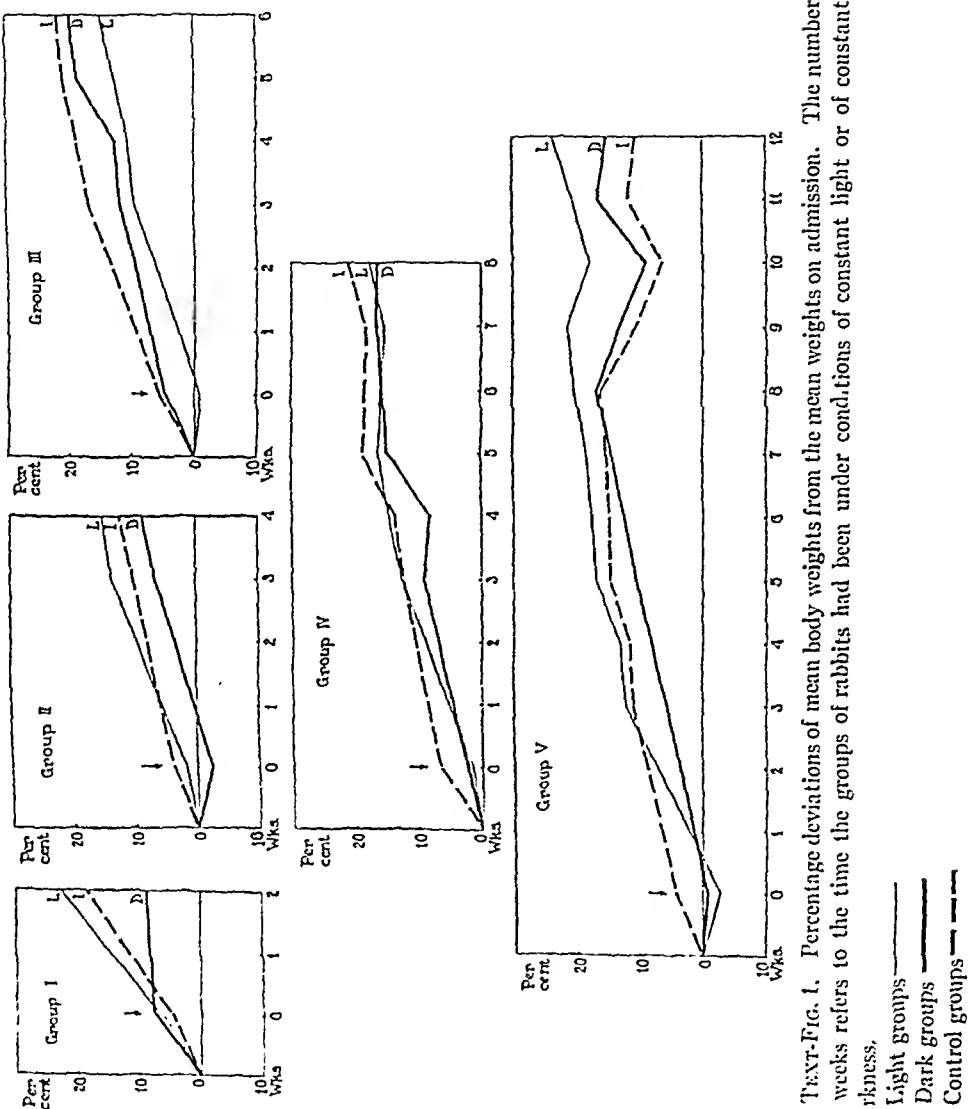
On December 31, 1924 and January 2, 1925, 50 rabbits were placed in the light room, 50 in the dark room, while the third lot of 50 animals remained in the control or unaltered animal room. Groups of 10 rabbits from each room were killed and examined 2, 4, 6, 8, and 12 weeks later.¹ In addition, other groups of normal

¹ Data for a 10 week period were obtained from additional groups of rabbits placed under the same conditions on January 29 and killed on April 8,

tion of the experiment, that is, of exposure to constant illumination or to constant exclusion of light, was 2 to 12 weeks. The physical state of all rabbits at the beginning of the experiment was excellent and it continued to be so in the case of the animals in the light room and those in the unaltered or control room. Although the same was generally true for the rabbits caged under conditions of constant darkness there were some indications that this environment was less favorable.

Evidence for the satisfactory condition of the rabbits is furnished by body weight curves (Text-fig. 1) in which the average or mean value for each group of 10 animals is used. The changes in weight are expressed in percentage deviations from the average weight of the group on admission. The curves show that, on the whole, each group of rabbits gained in weight steadily and fairly uniformly. In Groups I and II (2 and 4 weeks exposure) the light room animals increased in weight more than the "inside controls," while those in the dark room gained the least. In Group III (6 weeks exposure) the inside controls and the dark room animals gained more than the light room rabbits. In Group IV (8 weeks exposure) the gain of all three sets approximated each other with a tendency of those in the dark room to gain the least and the inside controls to gain the most; the light room rabbits of this group showed a greater increase in weight during the first weeks than those in the dark room but toward the end of the experiment the two curves are practically identical. In the last group (12 weeks exposure) the greatest increase in body weight was made by the group of light room rabbits, while the inside controls gained the least. The dark room animals showed the smallest gains for the first 7 weeks, but during the last month they slightly exceeded in this particular the inside controls. It should be mentioned that this group of inside controls was not entirely comparable to the others in that it contained three older and larger rabbits. During the first 2 months, these animals gained slightly in weight but in the subsequent month lost weight, which largely contributed to the disproportionate drop in the average body weight of the group during the latter part of the experiment.

Reference to the curves brings out the fact that, except with Group I, the mean body weights of the various groups at the time the rabbits were placed in the light and dark rooms were not uniform. That is,



TEXT-FIG. 1. Percentage deviations of mean body weights from the mean weights on admission. The number of weeks refers to the time the groups of rabbits had been under conditions of constant light or of constant darkness.

Light groups —
Dark groups - - -
Control groups . . .

good state of nutrition. The appetite of the rabbits in the light room was noticeably increased, especially during the first 6 weeks of the experiment, and it was found that they ate more food than the controls. The appetite of the dark room rabbits, on the other hand, showed no apparent deviation from the normal.

It is also of interest to note that the general behavior of the rabbits was not apparently disturbed by living in an environment of constant light. They did not try to avoid looking at the arcs and lamps and, as far as could be determined, slept as usual. During the first weeks of the experiment the animals seemed more alert and lively but this condition did not persist. On the other hand, there was nothing in the behavior of the rabbits in the dark room that was peculiar to this group of animals.

The health of the animals was, in general, excellent. Few instances of spontaneous disease developed during the period of observation that were recognizable clinically.

There were 2 cases of ear canker, 1 each among the light room and inside control rabbits; 1 instance of a small, encapsulated, subcutaneous abscess in an inside control animal, and 3 cases of clinical snuffles, all among the inside controls.

It was found by postmortem examination, however, that 59.3 per cent of the rabbits in these groups (light room 60 per cent, dark room 58 per cent, inside controls 60 per cent) showed gross lesions of some kind but in only a few cases were these more than slight or moderate in degree.

The great majority of the lesions, which were distributed fairly uniformly among the subgroups, were scarring of the kidneys and liver, but there was 1 example of a marked chronic coccidiosis of the liver with recent lesions, and 20 instances of a purulent infection of the cranial sinuses, middle ear, or mastoids. Other conditions noted included slight degenerations of the aorta, verminous infections of the intestinal tract, coccidial cysts of the abdominal cavity not associated with visible lesions of the liver, 1 case of a healed pulmonary abscess, 1 instance of a healing abscess of the myocardium and another of hypertrophy of the right ventricle of undetermined origin, 1 case of marked malformation of the kidneys, 1 of a parathyroid cyst, 2 axillary lymph node cysts, and 2 instances of hydrocephalus.

As a further indication of the health of the animal the condition of body fat was noted at autopsy in such general terms as excessive,

from the time of admission to the beginning of the experiment, some groups gained less weight than others, while some actually lost weight. A more accurate idea of the effect of the experimental environments upon general physical conditions as indicated by the criterion of body weight is obtained by comparing the final weight values with those at the beginning of the experimental period (Table I). All the control groups showed an increase in weight; the gain of the fifth group was much smaller than the others owing, as has been mentioned above, to the irregularity of the group in respect to the age and size of the animals. The greatest gains in weight were made by the rabbits

TABLE I.
Increase in Mean Body Weight during Experimental Period.

	Initial weights			Final weights			Actual and percentage gain					
	Light room	Dark room	Controls	Light room	Dark room	Controls	Light room		Dark room		Controls	
	gm.	gm.	gm.	gm.	gm.	gm.	gm.	per cent	gm.	per cent	gm.	per cent
Group I.....	2200	2125	2198	2528	2190	2433	328	14.9	65	3.1	235	10.7
Group II.....	2178	2200	2095	2460	2450	2350	282	13.0	250	11.4	255	12.2
Group III.....	1995	2125	2225	2310	2415	2570	315	15.8	290	13.6	345	15.5
Group IV.....	2100	2225	2333	2433	2530	2653	333	15.9	305	13.7	320	13.7
Group V.....	2045	2035	2350	2603	2370	2530	558	27.3	335	16.5	150	6.3
Mean.....	2104	2142	2246	2467	2391	2507	363	17.3	249	11.6	261	11.6

kept in the light room, and this was true of all five groups. The dark room groups gained less than those in the light room and in the case of the first three groups, less than the inside controls. The percentage increase of the fourth groups of dark room and inside control animals was the same. The fifth group in the dark room showed a much greater increase than the inside controls due largely to the disproportionately high value of the latter group at the beginning of the experiment.

It should be mentioned, in connection with the body weight observations, that each rabbit was given all the food he would consume with the idea of supplying optimum conditions for the maintenance of a

fat in rabbits which presumably have not been living a sedentary life and have not necessarily been constantly provided with abundant food is obviously of less significance as an index of physical condition than it is in rabbits living a caged existence with an ample food supply.

DISCUSSION.

The health of the animals employed in an experiment of this type is of importance first, from the point of view of the general reaction to artificial environments, and second, because of the influence of physical condition upon the weights of organs. The rabbits comprising this experiment were all in apparent good health throughout the period of observation, such spontaneous disease as could be recognized clinically appearing unimportant.

Body weight observations have been used as an index to the effects of constant illumination or exclusion of light upon the general physical condition. There were five groups of 10 rabbits kept under each of these environmental conditions for 2, 4, 6, 8, and 12 weeks respectively. All groups gained in weight during these periods, but the greatest gains were made by the rabbits exposed to constant light. While there was also an increase in body weight of the groups kept in constant darkness it was less pronounced and more irregular than that of the groups in constant light and, on the whole, than that of the control rabbits kept in an ordinary animal room, a fact which suggests that the exclusion of light was relatively unfavorable.

At the end of the experimental period, all the rabbits appeared to be in good condition, but postmortem examination showed that the body fat of several was relatively scanty. The distribution of these animals, 3 in the light, 3 in the control, and 7 in the dark room groups, suggests that some impairment of nutrition may be related to prolonged caging in an absence of light. This is further suggested by the fact that 4 of the 7 dark room rabbits were of the group which had been kept in this environment for 12 weeks. Gross lesions of some kind were found in 11 of the 13 rabbits with scant body fat, those of the kidneys appearing most significant. The kidneys were affected in 9 instances and in 4 of these the lesions appeared active, the organs being enlarged, soft, opaque, and either markedly congested or yellow. To what extent inactive lesions of the kidneys, with moderate or

abundant, moderate, scant, or none. The body fat was either scanty or practically absent in only 13 rabbits (8.06 per cent) distributed as follows: 3 in the light, 7 in the dark, and 3 in the inside control groups respectively.

A number of gross lesions were found at autopsy in all save 2 of these 13 rabbits.

Of the 3 light room rabbits, there were 2 with active lesions of the kidneys, 1 with a purulent inflammation of both mastoids, and all 3 showed healed or inactive coccidiosis of the liver of slight degree. Among the 7 dark room rabbits, there were 5 with kidney lesions, 1 of which was active and 4 inactive with varying degrees of scarring; there was 1 instance of an active and 1 of a slight and inactive coccidiosis, and 1 of a purulent exudate in the nasal passages and mastoids. Among the 3 inside control rabbits, 2 showed kidney lesions, of which 1 was active and the other inactive with marked scarring; 1 animal had a slight active and 1 a slight inactive coccidiosis, and another had a purulent inflammation of the nasal passages and cranial sinuses. There was some degree of thymic mass depletion in 9 and an enlargement of the spleen in 10 of these 13 rabbits.

The 40 rabbits comprising the four groups of outside controls (recently acquired animals) were clinically free from any evidence of disease but there was practically the same incidence of visible lesions at autopsy as in the other groups, that is, 57.5 per cent.

In the case of 20 normal rabbits killed on March 12, 1925, midway between the fourth and fifth experimental groups, the incidence of postmortem lesions was 60 per cent. The types of lesion did not differ from those found in the light, dark, and inside control rabbits, the majority being slight or moderate and principally affecting the same organs. Among the outside controls there were 2 cases of a marked active coccidiosis of the liver and 5 instances of a moderate or marked scarring of the kidneys. In the March group, the incidence of active coccidiosis of considerable extent was higher—5 of the 20 rabbits showing this condition; in 2 animals there was marked scarring of the kidneys. In none of the outside controls was there any purulent inflammation about the head, while 2 of the March normals showed this condition. The body fat of 3 outside control rabbits was scanty. In each animal the thymic mass was small and the spleen enlarged, but only 1 showed gross lesions of probable significance, namely, a possibly active lesion of the kidneys and an active coccidiosis of the liver. A similar state of body fat occurred in 6 of the 20 March normals, 4 of which showed a small thymus and 3 of these a slightly enlarged spleen. There was 1 instance of an active and another of an inactive coccidiosis, 1 of a purulent inflammation of the mastoids, while in 3 rabbits no gross lesions were found. The occurrence of relatively little

in organ weight as one passes from rabbits with slight or moderate lesions to those with marked lesions, particularly in relation to the activity of such conditions. The number of marked or active lesions was extremely few in this series of rabbits, and it has been found by actual determination that these conditions have not essentially affected the mean organ values employed in analyzing the experimental results obtained, except in certain particular instances, as in the case of the deep cervical lymph nodes, a marked enlargement of which is associated with a purulent infection of the cranial sinuses. The effect of an impaired state of health upon organ weight does not have to be considered in the present connection as all the rabbits of the experiment were in good physical condition during the period of observation.

SUMMARY AND CONCLUSIONS.

A group of 50 normal male rabbits kept under conditions of constant light that had none of the shorter ultra-violet rays and another group kept in constant darkness for 2 to 12 weeks were observed clinically and subjected to postmortem examination for the purpose of determining the effect of these environmental conditions upon general body health and the weights of organs. A similar group of 50 rabbits caged in an ordinary animal room for the same period, and two groups of 40 and 20 rabbits respectively, which had recently been brought into the laboratory, served as controls.

It was found that the general health of the rabbits was not impaired by the artificial light or the exclusion of light. The gain in body weight which occurred in all groups was especially marked in the case of those kept under conditions of constant light.

The incidence of spontaneous disease recognizable clinically during the experiment was extremely low and of a mild character and did not obviously disturb the health of the animal. It was found at post-mortem examination, on the other hand, that 59.3 per cent of the rabbits caged indoors, that is in the light, dark, or unaltered rooms, and 58.3 per cent of those recently brought to the laboratory had visible lesions of some kind. The great majority of these lesions, however, were of a slight grade, and none appeared to have any deleterious effects upon the general physical state of the animals.

marked scarring, are associated with scant body fat is not known, but such was the condition in 5 rabbits. On the other hand, moderate or marked renal scarring is not necessarily accompanied by scant body fat, for there were 19 other rabbits among the 150 experimental animals with such lesions but with no evident fat reduction.

The thymic mass in the rabbit has been found to be a fairly delicate indicator of general physical condition in that its size, consistency, and the amount of associated fat are roughly proportional to the state of nutrition of the animal. Among the 13 rabbits of the experimental series which showed a scant amount of body fat, the thymic mass was considered to be depleted to a greater or less extent in 9 animals, 5 of which were in the dark room groups.

Enlargement of the spleen was frequently seen in the entire series of rabbits and no relationship could be made out between its occurrence and the presence or character of gross lesions, or the condition of the body fat.

At postmortem examination it was found that visible lesions of some kind were present in 59.3 per cent of the 150 rabbits caged under conditions of constant illumination, of constant darkness, and of an ordinary animal room. Lesions of a similar type and distribution were found in 57.5 per cent of one group of 40, and in 60 per cent of a group of 20 rabbits recently brought to the laboratory. These figures are higher than those of two groups of 350 and 295 normal rabbits examined during $3\frac{1}{2}$ years, namely, 40.9 and 53.2 per cent (3). While the larger number of animals in these two series suggests a possible explanation for these differences, the uniform incidence of lesions among the four major groups of this experiment indicates a higher proportion of pathological change in the rabbit stock at this particular time. The state of the lesions found in the experimental animals did not suggest that the conditions of constant light or of constant darkness were associated with heightened activity of the pathological process.

It has been found from the observations of the two large series of normal rabbits referred to, that as long as the animals remain in good health the values obtained for organ weights of animals with lesions do not differ materially from those of animals that are entirely free from lesions (3, 4). There is a slight tendency toward an increase

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nally, results are given for 20 normal rabbits examined on March 12, 1925, midway in time between the fourth and fifth experimental groups.

COMMENT.

This portion of the paper is concerned mainly with the data obtained. An analysis of the organ weights contained in Tables I and II will be taken up in Paper III (3).

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EFFECTS OF LIGHT ON NORMAL RABBITS, WITH ESPECIAL REFERENCE TO THE ORGANIC REACTION.

II. ORGAN WEIGHTS.

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(Received for publication, June 11, 1926.)

In Paper I of this series (1), it was stated that the purpose of our investigation was the testing by experimental means, of whether there is a relationship between the nature or character of the light environment, on the one hand, and the physical state and functional activities of the animal organism, on the other. The methods employed and the general clinical observations and postmortem findings have been described. The present paper is concerned with the effects of light upon organ weights.

RESULTS.

The weights of the organs are presented in tabular form. The actual weights are given in Table I and the relative weights, or the weights per kilo of net body weight, in Table II. In both tables the order of organs and of animal groups is the same. For comparison, observations obtained from a series of 350 normal rabbits are given first (2).¹ Then follow the results of this experiment in the order of light and dark room animals and inside and outside controls. With the exception of the outside controls, these major divisions are divided into five groups, comprising 10 rabbits each, and corresponding to the 2, 4, 6, 8, and 12 week periods of exposure to the environments of the experiment. There are only four groups of outside controls. Fi-

¹ In the case of the gross body weight, the kidneys (actual), testicles (relative), suprarenals (actual), and axillary lymph nodes (actual), the figures given for the 350 normal rabbits differ in a few respects from those in the original paper. The changes represent corrections of slight errors in the original figures.

TABLE I—*Continued.*

Organ	Group	Arithmetical mean	Minimum	Maximum	Standard deviation	Probable error	Coefficient of variation
		<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	\pm <i>gm.</i>	\pm <i>gm.</i>	<i>per cent</i>
Net body weight	N I	1848.5	1150	3010	323.3	218.0	17.49
	L 1	2074.0	1780	2755	287.6	193.98	13.87
	2	2048.0	1450	2585	358.6	241.86	17.52
	3	1985.0	1760	2235	157.7	106.37	7.95
	4	2094.0	1805	2500	232.0	156.48	11.08
	5	2188.0	1756	2730	304.9	205.66	13.93
	D 1	1756.0	1440	2035	210.8	142.20	12.00
	2	1988.0	1795	2260	150.8	101.71	7.58
	3	1994.0	1765	2395	175.4	118.31	8.79
	4	2102.0	1860	2505	213.4	143.94	10.16
	5	1930.0	1613	2415	267.0	180.10	13.83
	I 1	2009.0	1695	2440	198.1	133.62	9.86
	2	1913.0	1765	2175	126.8	85.53	6.63
	3	2142.0	1885	2350	124.4	83.91	5.81
	4	2194.0	1955	2500	198.1	133.60	9.03
	5	2112.0	1715	2660	327.9	221.20	15.53
	O 1	1718.0	1395	2135	247.5	166.94	14.41
	2	1902.0	1477	2505	306.9	207.00	16.14
	3	1866.0	1505	2485	305.2	205.86	16.36
	4	1711.0	1455	2190	211.9	142.93	12.38
	M	1641.0	1380	2020	184.7	124.59	11.26
Heart	N I	5.26	3.20	10.18	0.96	0.64	18.23
	L 1	5.90	4.86	7.70	0.898	0.606	15.22
	2	5.97	4.40	9.24	1.29	0.87	21.61
	3	5.38	4.50	6.70	0.719	0.485	13.34
	4	5.22	4.25	6.42	0.706	0.476	13.52
	5	6.02	4.85	7.85	0.90	0.61	15.02
	D 1	5.40	4.40	6.54	0.69	0.47	12.78
	2	5.55	4.67	6.82	0.71	0.48	12.79
	3	5.33	4.50	6.55	0.58	0.39	10.84
	4	5.52	4.45	6.85	0.72	0.48	12.97
	5	5.30	4.50	6.65	0.68	0.46	12.90
	I 1	5.81	4.65	6.64	0.64	0.43	10.95
	2	5.66	4.45	8.05	0.96	0.65	16.93
	3	5.54	4.61	6.95	0.65	0.44	11.81
	4	6.19	5.10	7.65	0.882	0.594	14.25
	5	5.86	4.30	7.43	0.77	0.52	13.05
	O 1	5.40	3.86	7.30	0.89	0.60	16.48
	2	6.05	4.63	8.00	0.90	0.61	14.86
	3	5.76	3.95	7.50	1.07	0.72	18.58
	4	5.15	4.40	7.40	0.90	0.61	17.50
	M	4.87	3.55	6.55	0.604	0.41	12.39

TABLE I.

Results for Body and Actual Organ Weights According to Groups.

Organ	Group	Arithmetical mean	Minimum	Maximum	Standard deviation	Probable error	Coefficient of variation
		gm.	gm.	gm.	± gm.	± gm.	per cent
Gross body weight	N I	2265	1400	3500	352.8	237.90	15.57
	L 1	2528	2200	3325	323.7	218.33	12.81
	2	2460	1850	3000	375.5	253.28	15.26
	3	2310	2050	2650	194.0	130.85	8.40
	4	2433	2125	2875	247.5	176.94	10.18
	5	2603	2100	3225	373.5	251.93	14.35
	D 1	2188	1825	2475	210.7	142.10	9.63
	2	2460	2200	2750	179.3	120.90	7.28
	3	2415	2150	2975	229.7	154.93	9.51
	4	2530	2275	3000	236.3	159.40	9.33
	5	2358	1925	2825	309.2	208.60	13.11
	I 1	2478	2150	3050	248.1	167.34	10.01
	2	2350	2175	2650	137.8	92.95	5.86
	3	2570	2350	2775	147.3	99.35	5.73
	4	2618	2275	2950	249.5	168.30	9.53
	5	2545	2075	3100	353.9	238.70	13.90
	O 1	2235	1800	2600	246.2	166.06	11.02
	2	2440	2050	3200	321.1	216.58	13.16
	3	2313	1950	2950	313.5	211.46	13.56
	4	2158	1800	2725	240.3	162.08	11.14
	M	2024	1725	2450	208.9	140.93	10.32
Gastrointes- tinal mass	N I	416.4	115	710	80.9	54.5	19.42
	L 1	454.0	340	570	66.1	45.58	14.56
	2	413.0	327	505	59.1	39.86	14.30
	3	325.0	230	415	63.2	42.63	19.45
	4	338.0	260	402	44.7	30.15	13.23
	5	415.0	325	562	80.2	50.06	19.31
	D 1	432.0	375	485	38.8	26.17	8.99
	2	473.0	325	640	89.1	60.06	18.84
	3	421.0	295	580	76.4	51.53	18.14
	4	428.0	305	495	48.8	32.9	11.40
	5	427.0	305	575	83.1	56.05	19.45
	I 1	469.0	335	610	81.9	53.24	17.46
	2	438.0	305	555	82.1	53.35	18.74
	3	428.0	337	532	56.4	38.04	13.18
	4	432.0	305	565	77.2	52.1	17.87
	5	434.0	360	555	57.6	38.8	13.28
	O 1	501.0	365	695	91.4	61.65	18.24
	2	538.0	400	740	101.5	70.49	19.42
	3	447.0	355	507	33.8	22.8	7.56
	4	446.0	345	570	67.5	45.53	15.14
	M	383.0	195	500	64.4	43.42	16.81

TABLE I—*Continued.*

Organ	Group	Arithmetical mean	Minimum	Maximum	Standard deviation	Probable error	Coefficient of variation
		<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	\pm <i>gm.</i>	\pm <i>gm.</i>	<i>per cent</i>
Spleen	N I	0.990	0.08	3.20	0.436	0.294	44.04
	L 1	1.344	0.89	2.28	0.425	0.287	31.62
	2	1.209	0.64	1.90	0.408	0.275	33.75
	3	1.005	0.40	2.18	0.512	0.345	50.95
	4	0.978	0.355	2.035	0.421	0.284	44.06
	5	1.270	0.095	2.35	0.658	0.444	51.81
	D 1	1.314	0.50	1.92	0.405	0.273	30.82
	2	1.154	0.745	1.705	0.378	0.255	32.75
	3	1.400	0.80	2.82	0.547	0.369	39.07
	4	1.021	0.575	1.535	0.286	0.193	28.01
	5	1.195	0.65	2.285	0.510	0.344	42.67
	I 1	1.594	0.88	3.33	0.719	0.485	44.48
	2	1.273	0.59	2.575	0.525	0.354	41.24
	3	1.112	0.715	2.15	0.417	0.281	37.50
	4	1.108	0.56	1.715	0.331	0.223	29.84
	5	1.309	0.87	2.685	0.537	0.362	41.02
	O 1	1.193	0.71	1.67	0.270	0.182	22.63
	2	1.104	0.55	1.94	0.374	0.252	33.88
	3	1.433	0.56	3.00	0.665	0.449	46.41
	4	1.245	0.53	1.535	0.274	0.185	22.01
	M	1.110	0.47	2.17	0.462	0.312	41.60
Thymus	N I	2.210	0.45	5.90	0.848	0.572	38.37
	L 1	2.865	1.94	4.38	0.881	0.594	30.75
	2	2.378	1.54	3.70	0.759	0.512	31.92
	3	2.182	1.54	3.23	0.421	0.284	19.27
	4	2.671	1.86	4.25	0.687	0.464	25.73
	5	3.136	1.85	4.97	1.043	0.704	33.26
	D 1	2.527	0.91	3.78	0.784	0.529	31.03
	2	2.401	1.54	3.55	0.659	0.444	27.44
	3	2.350	0.82	3.40	0.680	0.459	28.93
	4	2.105	1.30	2.92	0.471	0.318	22.37
	5	2.213	0.90	3.90	0.959	0.647	43.33
	I 1	3.328	1.70	6.30	1.493	1.007	44.86
	2	2.577	1.26	3.50	0.655	0.442	25.42
	3	2.338	1.43	3.05	0.466	0.314	19.93
	4	2.464	0.63	4.10	0.937	0.632	38.04
	5	1.967	1.30	3.15	0.622	0.420	31.62
	O 1	1.670	0.97	3.85	0.906	0.611	54.25
	2	2.039	0.73	3.55	0.931	0.628	45.66
	3	2.028	1.17	3.00	0.562	0.379	27.71
	4	1.666	0.95	3.17	0.677	0.457	40.64
	M	2.260	0.80	3.50	0.851	0.574	37.65

TABLE I—*Continued.*

Organ	Group	Arithmetical mean	Minimum	Maximum	Standard deviation	Probable error	Coefficient of variation
		<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>≠ gm.</i>	<i>≠ gm.</i>	<i>per cent</i>
Liver	N I	84.3	50.0	150.0	19.7	13.3	23.39
	L 1	114.4	95.0	140.0	12.46	6.88	10.88
	2	70.6	57.0	82.0	7.78	5.25	11.02
	3	79.8	67.0	100.0	9.67	6.52	12.12
	4	74.7	60.0	100.0	12.80	8.63	17.14
	5	77.3	55.0	105.0	13.92	9.39	18.01
	D 1	99.5	78.0	111.0	8.86	5.98	8.90
	2	97.2	65.0	122.0	14.50	9.78	14.91
	3	101.8	80.0	135.0	18.7	12.6	18.36
	4	109.0	73.0	155.0	23.08	15.57	21.17
	5	110.9	85.0	145.0	17.8	12.01	16.05
	I 1	92.6	74.0	115.0	11.99	8.09	12.95
	2	99.1	85.0	118.0	10.90	7.35	10.99
	3	80.1	63.0	95.0	9.9	6.68	12.46
	4	95.4	65.0	127.0	18.45	12.24	19.34
	5	94.7	67.0	120.0	16.86	11.37	17.81
	O 1	106.2	57.0	185.0	32.7	22.06	30.89
	2	85.6	67.0	122.0	15.1	10.19	17.64
	3	73.3	55.0	100.0	11.7	7.89	15.96
	4	80.6	55.0	120.0	19.7	13.29	24.44
	M	89.9	55.0	120.0	15.04	10.15	16.73
Kidneys	N I	12.7	7.15	22.73	2.14	1.44	16.85
	L 1	15.44	13.20	18.50	1.84	1.24	11.92
	2	13.59	9.55	21.25	3.11	2.10	22.88
	3	12.16	10.92	13.25	0.797	0.54	6.55
	4	12.55	11.05	15.60	1.40	0.95	11.18
	5	13.21	8.80	16.68	1.84	1.24	13.92
	D 1	14.00	10.85	17.80	2.13	1.44	15.21
	2	14.60	11.40	17.30	1.99	1.34	13.63
	3	14.28	11.80	16.55	1.44	0.97	10.04
	4	13.86	10.80	17.04	1.92	1.29	13.85
	5	12.61	9.20	14.42	1.58	1.07	12.52
	I 1	15.25	13.45	17.67	1.19	0.80	7.80
	2	14.01	10.37	16.90	1.91	1.29	13.63
	3	14.05	11.90	16.34	1.27	0.86	9.04
	4	13.89	11.60	19.40	2.33	1.57	16.78
	5	13.51	9.25	19.97	2.84	1.91	21.01
	O 1	13.46	9.65	17.56	2.26	1.52	16.80
	2	14.25	11.32	17.85	1.98	1.34	13.89
	3	13.80	11.50	19.18	2.24	1.51	16.23
	4	12.98	10.00	15.10	1.47	0.99	11.33
	M	13.20	10.90	16.70	1.53	1.03	11.56

TABLE I—*Continued.*

Organ	Group	Arithmetical mean	Minimum	Maximum	Standard deviation	Probable error	Coefficient of variation
		gm.	gm.	gm.	± gm.	± gm.	per cent
Thyroid	N I	0.23048	0.085	1.570	0.16209	0.10933	70.33
	L 1	0.2055	0.120	0.305	0.06109	0.04121	29.72
	2	0.1835	0.120	0.260	0.03772	0.02544	20.56
	3	0.1950	0.150	0.280	0.0344	0.0232	17.64
	4	0.2600	0.155	0.375	0.0629	0.04243	24.19
	5	0.2550	0.160	0.355	0.0668	0.04506	26.20
	D 1	0.1880	0.125	0.330	0.0536	0.0362	28.51
	2	0.2219	0.150	0.330	0.0483	0.0326	21.76
	3	0.2467	0.185	0.315	0.0443	0.0299	17.95
	4	0.2395	0.160	0.340	0.0536	0.0362	22.37
	5	0.2545	0.160	0.810	0.1866	0.1259	73.32
		(0.1928)	(0.160)	(0.245)	(0.0252)	(0.017)	(13.07)
	I 1	0.2459	0.165	0.380	0.06304	0.04252	25.64
	2	0.2657	0.163	0.397	0.0638	0.0430	24.01
	3	0.3180	0.180	0.512	0.0899	0.0606	28.27
		(0.2964)	(0.180)	(0.385)	(0.06608)	(0.0445)	(22.94)
	4	0.4115	0.208	0.935	0.2424	0.1635	58.90
		(0.2657)	(0.208)	(0.310)	(0.0426)	(0.0287)	(16.02)
	5	0.3082	0.190	0.390	0.0695	0.0469	22.55
	O 1	0.2196	0.107	0.520	0.1089	0.0735	49.59
		(0.1862)	(0.107)	(0.255)	(0.0451)	(0.0304)	(24.22)
	2	0.1880	0.115	0.280	0.0479	0.0323	25.48
	3	0.1852	0.115	0.410	0.0439	0.0296	23.70
	4	0.2540	0.110	0.480	0.1231	0.0830	48.46
		(0.2289)	(0.110)	(0.410)	(0.1025)	(0.0690)	44.77
	M	0.2622	0.120	1.730	0.3403	0.2296	129.70
		(0.1852)	(0.120)	(0.340)	(0.0510)	(0.0344)	(27.54)
Parathyroids	N I	0.01256	0.002	0.035	0.00486	0.00328	38.69
	L 1	0.01260	0.008	0.015	0.00220	0.00148	17.46
	2	0.01020	0.006	0.019	0.00329	0.00222	32.24
	3	0.00970	0.006	0.016	0.00290	0.00196	29.90
	4	0.00950	0.004	0.013	0.00253	0.00171	26.63
	5	0.01170	0.007	0.020	0.00387	0.00261	33.08
	D 1	0.01300	0.007	0.018	0.00355	0.00239	27.30
	2	0.01080	0.005	0.015	0.00309	0.00208	28.61
	3	0.01310	0.008	0.018	0.00270	0.00182	20.61
	4	0.01160	0.006	0.022	0.00480	0.00324	41.37
	5	0.01330	0.005	0.024	0.00655	0.00442	49.24
	I 1	0.01200	0.009	0.020	0.00328	0.00221	26.89
	2	0.01260	0.007	0.022	0.00412	0.00278	32.70
	3	0.01310	0.008	0.025	0.00452	0.00305	34.50
	4	0.01222	0.006	0.019	0.00394	0.00266	32.22
	5	0.01840	0.013	0.027	0.00500	0.00338	27.20

TABLE I—*Continued.*

Organ	Group	Arithmetical mean	Minimum	Maximum	Standard deviation	Probable error	Coefficient of variation
		<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	\pm <i>gm.</i>	\pm <i>gm.</i>	<i>per cent</i>
Testicles	N I	4.86	0.81	9.12	1.63	1.10	33.54
	L 1	5.08	4.40	6.33	0.57	0.39	11.20
	2	5.65	3.55	8.50	1.38	0.93	24.42
	3	5.07	4.15	6.70	0.68	0.46	13.31
	4	5.25	3.70	6.89	0.99	0.67	18.86
	5	5.23	4.05	6.43	0.67	0.45	12.72
	D 1	4.92	3.73	7.15	0.995	0.67	20.22
	2	5.39	3.25	6.90	0.904	0.61	16.77
	3	5.77	4.30	7.17	0.87	0.59	15.06
	4	5.31	3.90	6.60	0.94	0.63	17.70
	5	4.57	2.55	6.20	1.07	0.72	23.41
	I 1	5.50	3.90	7.72	1.04	0.70	18.91
	2	5.23	3.88	6.88	1.10	0.74	21.05
	3	6.06	4.98	8.05	0.92	0.62	15.13
	4	5.88	4.55	7.35	0.97	0.66	16.51
	5	4.81	3.28	6.90	0.99	0.67	20.58
	O 1	5.79	1.77	9.01	2.23	1.50	38.51
	2	5.13	3.13	7.10	1.51	1.02	29.44
	3	5.05	1.75	8.69	2.10	1.42	41.58
	4	4.53	3.40	6.30	0.86	0.58	18.92
	M	4.27	2.75	7.20	1.08	0.73	25.32
Brain	N I	9.31	7.42	12.03	0.78	0.526	8.37
	L 1	9.34	8.10	10.00	0.59	0.40	6.28
	2	9.56	8.65	10.45	0.49	0.33	5.15
	3	8.67	7.51	9.75	0.77	0.52	8.99
	4	8.84	8.15	9.70	0.51	0.34	5.74
	5	9.73	8.05	10.90	0.81	0.54	8.28
	D 1	9.60	8.75	10.50	0.62	0.42	6.48
	2	9.22	8.00	10.43	0.71	0.48	7.65
	3	9.46	8.40	10.39	0.674	0.454	7.12
	4	9.10	8.10	10.75	0.80	0.54	8.80
	5	9.08	7.35	10.00	0.73	0.49	8.05
	I 1	9.49	7.57	11.00	0.86	0.58	9.10
	2	9.21	8.00	10.24	0.68	0.46	7.33
	3	9.49	7.90	11.33	0.94	0.63	9.90
	4	9.24	8.65	9.82	0.373	0.251	4.04
	5	9.35	8.25	10.20	0.596	0.40	6.38
	O 1	9.27	8.00	10.00	0.64	0.43	6.87
	2	9.62	8.07	11.30	0.92	0.62	9.58
	3	9.39	8.19	10.45	0.58	0.39	6.16
	4	8.90	8.10	9.45	0.46	0.31	5.15
	M	8.76	8.10	10.05	0.60	0.40	6.84

TABLE I—*Continued.*

Organ	Group	Arithmetical mean	Minimum	Maximum	Standard deviation	Probable error	Coefficient of variation
		<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	\pm <i>gm.</i>	\pm <i>gm.</i>	<i>per cent</i>
Suprarenals— Continued	O 1	0.413	0.250	0.760	0.1627	0.1097	39.29
	2	0.430	0.198	0.720	0.1921	0.1296	44.67
	3	0.419	0.192	0.710	0.1593	0.1075	38.01
	4	0.333	0.145	0.640	0.1604	0.1082	48.17
	M	0.269	0.138	0.530	0.1018	0.0687	37.83
Pineal	N I	0.01609	0.005	0.030	0.00429	0.00289	26.66
	L 1	0.01330	0.010	0.016	0.00205	0.00138	15.41
	2	0.01240	0.008	0.016	0.00280	0.00189	22.58
	3	0.01290	0.006	0.017	0.00401	0.00271	31.09
	4	0.01260	0.007	0.019	0.00349	0.00235	27.38
	5	0.01360	0.011	0.017	0.00174	0.00107	12.79
	D 1	0.01430	0.009	0.023	0.00395	0.00266	27.62
	2	0.01270	0.010	0.016	0.00205	0.00138	16.12
	3	0.01620	0.008	0.022	0.00384	0.00259	23.70
	4	0.01540	0.010	0.023	0.00436	0.00294	28.31
	5	0.01440	0.008	0.021	0.00366	0.00247	25.41
	I 1	0.01510	0.010	0.022	0.00381	0.00257	25.23
	2	0.01580	0.012	0.020	0.00302	0.00204	19.11
	3	0.01450	0.005	0.020	0.00366	0.00245	25.24
	4	0.01450	0.008	0.021	0.00432	0.00291	29.78
	5	0.01690	0.010	0.023	0.00396	0.00267	23.44
	O 1	0.01366	0.010	0.020	0.00380	0.00256	27.74
	2	0.01430	0.011	0.027	0.00447	0.00302	31.25
	3	0.01320	0.005	0.025	0.00563	0.00380	42.65
	4	0.01300	0.008	0.020	0.00434	0.00293	31.08
	M	0.01345	0.005	0.018	0.00347	0.00234	25.80
Popliteal lymph nodes	N I	0.25539	0.085	0.720	0.08241	0.05559	32.27
	L 1	0.273	0.170	0.400	0.0682	0.0460	24.98
	2	0.239	0.180	0.350	0.0482	0.0325	20.17
	3	0.242	0.170	0.350	0.0463	0.03123	19.13
	4	0.236	0.145	0.430	0.0857	0.05781	37.10
	5	0.241	0.145	0.350	0.0585	0.03946	24.27
	D 1	0.277	0.200	0.370	0.0487	0.0329	17.61
	2	0.292	0.220	0.360	0.0498	0.0336	17.08
	3	0.231	0.105	0.350	0.0661	0.0446	28.61
	4	0.248	0.155	0.330	0.0462	0.0312	18.62
	5	0.191	0.100	0.310	0.0666	0.0449	34.86
	I 1	0.313	0.200	0.405	0.0568	0.0383	18.15
	2	0.250	0.150	0.350	0.0576	0.0389	23.04
	3	0.254	0.165	0.420	0.0646	0.0436	25.43
	4	0.251	0.180	0.295	0.0414	0.0279	16.49
	5	0.198	0.090	0.350	0.0682	0.0460	34.43

TABLE I—*Continued.*

Organ	Group	Arithmetical mean	Minimum	Maximum	Standard deviation	Probable error	Coefficient of variation
		<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	\pm <i>gm.</i>	\pm <i>gm.</i>	<i>per cent</i>
Parathyroids —Continued	O 1	0.01460	0.008	0.033	0.00687	0.00463	47.05
	2	0.01230	0.007	0.027	0.00558	0.00376	45.37
	3	0.01170	0.004	0.016	0.00304	0.00206	25.98
	4	0.00850	0.003	0.014	0.00323	0.00218	38.00
	M	0.01070	0.006	0.017	0.00357	0.00241	33.32
Hypophysis	N I	0.0280	0.015	0.044	0.00517	0.00349	18.46
	L 1	0.0272	0.017	0.045	0.00802	0.00541	29.49
	2	0.0291	0.018	0.045	0.00664	0.00448	22.82
	3	0.0248	0.022	0.028	0.00183	0.00123	7.38
	4	0.0253	0.019	0.032	0.00349	0.00235	13.80
	5	0.0251	0.020	0.033	0.00353	0.00238	14.06
	D 1	0.0276	0.022	0.039	0.00471	0.00318	17.07
	2	0.0278	0.023	0.042	0.00526	0.00355	18.92
	3	0.0293	0.025	0.035	0.0034	0.00229	11.60
	4	0.0237	0.016	0.038	0.0058	0.00391	24.47
	5	0.0263	0.020	0.031	0.00363	0.00245	13.80
	I 1	0.0281	0.021	0.037	0.00441	0.00298	15.69
	2	0.0306	0.022	0.040	0.00574	0.00387	18.76
	3	0.0283	0.022	0.037	0.00392	0.00264	13.85
	4	0.0295	0.022	0.038	0.00554	0.00373	18.77
	5	0.0291	0.022	0.038	0.00505	0.00341	17.35
	O 1	0.0282	0.023	0.037	0.00376	0.00254	13.33
	2	0.0276	0.018	0.037	0.00514	0.00347	18.62
	3	0.0279	0.015	0.040	0.00665	0.00449	23.84
	4	0.0254	0.020	0.032	0.00366	0.00247	14.41
	M	0.0279	0.020	0.035	0.0038	0.00256	13.64
Suprarenals	N I	0.3858	0.115	1.050	0.1546	0.10428	40.07
	L 1	0.373	0.145	0.630	0.1383	0.09328	37.13
	2	0.478	0.280	1.112	0.2289	0.15439	47.89
	3	0.507	0.270	0.950	0.2198	0.14826	43.35
	4	0.425	0.346	0.517	0.0576	0.03885	13.55
	5	0.471	0.310	0.620	0.0939	0.06334	19.94
	D 1	0.317	0.180	0.485	0.0843	0.0569	26.55
	2	0.435	0.230	0.580	0.1091	0.0736	25.08
	3	0.395	0.260	0.650	0.1384	0.0934	35.03
	4	0.438	0.270	0.680	0.1330	0.0897	30.40
	5	0.498	0.250	0.940	0.2151	0.14508	43.20
	I 1	0.399	0.265	0.650	0.1107	0.0747	27.74
	2	0.426	0.180	0.865	0.1935	0.1305	45.42
	3	0.394	0.205	0.605	0.1088	0.0734	27.59
	4	0.487	0.255	0.750	0.1580	0.1063	32.39
	5	0.514	0.280	0.885	0.2180	0.1457	42.43

TABLE I—*Concluded.*

Organ	Group	Arithmetical mean	Minimum	Maximum	Standard deviation	Probable error	Coefficient of variation
		<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	\pm <i>gm.</i>	\pm <i>gm.</i>	<i>per cent</i>
Deep cervical lymph nodes—Continued	4	0.15200 (0.13100)	0.090 (0.090)	0.340 (0.160)	0.0658 (0.0210)	0.04440 (0.01400)	43.27 (15.68)
	5	0.22200 (0.18700)	0.100 (0.100)	0.540 (0.295)	0.1209 (0.0614)	0.08160 (0.04140)	54.48 (32.90)
	O 1	0.15000	0.085	0.235	0.0500	0.03400	32.20
	2	0.15200	0.110	0.195	0.0280	0.01900	18.49
	3	0.15000	0.070	0.285	0.0630	0.04200	41.67
	4	0.15300	0.075	0.360	0.0834	0.05600	54.51
	M	0.14900 (0.0120)	0.050 (0.050)	0.505 (0.220)	0.0494 (0.0435)	0.03300 (0.0293)	33.27 (36.32)
Mesenteric lymph nodes	N I	3.488	1.34	9.19	1.310	0.880	37.53
	L 1	3.160	1.80	4.25	0.673	0.454	21.30
	2	2.860	1.34	4.50	0.866	0.584	30.28
	3	2.998	2.02	4.50	0.655	0.442	21.85
	4	2.686	2.10	4.45	0.641	0.433	23.87
	5	2.950	1.60	4.60	0.909	0.613	30.48
	D 1	3.219	2.50	4.47	0.639	0.431	19.85
	2	2.950	1.71	3.68	0.674	0.455	22.84
	3	2.950	2.00	5.00	0.808	0.545	27.38
	4	2.680	2.35	3.10	0.206	0.139	7.68
	5	2.460	1.70	3.63	0.640	0.432	26.01
	I 1	3.235	2.20	4.85	0.798	0.538	24.73
	2	2.950	1.85	3.55	0.559	0.377	18.95
	3	3.290	2.45	4.45	0.565	0.381	17.17
	4	3.077	2.00	4.75	0.732	0.493	23.78
	5	3.020	2.00	4.15	0.695	0.468	23.01
	O 1	4.028	1.79	6.17	1.437	0.969	33.12
	2	5.680	2.40	11.47	2.460	1.659	43.31
	3	4.350	2.55	6.75	1.130	0.751	25.59
	4	4.550	3.23	5.75	0.796	0.537	17.50
	M	3.640	2.40	5.65	0.830	0.560	22.80

TABLE I—Continued.

Organ	Group	Arithmetical mean	Minimum	Maximum	Standard deviation	Probable error	Coefficient of variation
		<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	\pm <i>gm.</i>	\pm <i>gm.</i>	<i>per cent</i>
Popliteal lymph nodes—Continued	O 1	0.181	0.125	0.260	0.0459	0.0310	25.36
	2	0.254	0.135	0.390	0.0691	0.0466	27.21
	3	0.282	0.190	0.450	0.0664	0.0448	23.55
	4	0.233	0.160	0.410	0.0771	0.0520	32.66
	M	0.242	0.130	0.430	0.0977	0.0659	40.38
Axillary lymph nodes	N I	0.17585	0.040	0.400	0.06141	0.04142	34.92
	L 1	0.17300	0.105	0.240	0.04226	0.02850	24.83
	2	0.14600	0.085	0.300	0.06280	0.04236	43.01
	3	0.15500	0.080	0.305	0.05780	0.03899	37.29
	4	0.14500	0.100	0.235	0.04230	0.02853	29.17
	5	0.14200	0.080	0.220	0.03510	0.02668	24.72
	D 1	0.18750	0.130	0.270	0.03960	0.02670	21.12
	2	0.16200	0.065	0.215	0.04560	0.03080	28.14
	3	0.17000	0.070	0.240	0.05080	0.03430	29.88
	4	0.14280	0.085	0.180	0.03410	0.02300	23.87
	5	0.12650	0.075	0.165	0.03080	0.02080	24.34
	I 1	0.20200	0.095	0.280	0.05140	0.03470	25.45
	2	0.16000	0.125	0.230	0.03200	0.02170	20.06
	3	0.17800	0.105	0.240	0.04960	0.03350	27.92
	4	0.16400	0.085	0.210	0.04240	0.02860	25.83
	5	0.13700	0.100	0.180	0.02840	0.01910	20.73
	O 1	0.12400	0.090	0.170	0.03000	0.02000	24.29
	2	0.13900	0.090	0.205	0.03600	0.02400	25.91
	3	0.20400	0.112	0.365	0.07500	0.05100	36.91
	4	0.16300	0.110	0.265	0.04500	0.03000	27.36
	M	0.16600	0.070	0.485	0.09400	0.06300	56.22
Deep cervical lymph nodes	N I	0.15579	0.035	0.405	0.0732	0.04938	46.98
	L 1	0.13600	0.085	0.182	0.03202	0.02160	23.54
	2	0.12700	0.075	0.235	0.04399	0.02967	34.64
	3	0.14300	0.080	0.235	0.0525	0.03540	36.71
	4	0.10600	0.065	0.165	0.0314	0.02118	29.62
	5	0.23200	0.095	0.550	0.1604	0.10819	69.14
		(0.15400)	(0.095)	(0.250)	(0.0490)	(0.03320)	(31.87)
	D 1	0.16350	0.100	0.220	0.0435	0.02930	26.60
	2	0.18700	0.070	0.380	0.0838	0.05650	44.81
	3	0.15150	0.090	0.350	0.0712	0.04800	46.99
	4	0.11600	0.045	0.260	0.0549	0.03700	47.32
	5	0.13950	0.095	0.380	0.0815	0.05500	58.42
		(0.11300)	(0.095)	(0.140)	(0.0155)	(0.01040)	(13.72)
	I 1	0.19200	0.120	0.460	0.0954	0.06430	49.69
	2	0.14900	0.090	0.190	0.0317	0.02140	21.28
	3	0.13800	0.095	0.215	0.0343	0.02310	24.86

TABLE II—*Continued.*

Organ	Group	Arithmetical mean	Minimum	Maximum	Standard deviation	Probable error	Coefficient of variation
		<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	\pm <i>gm.</i>	\pm <i>gm.</i>	<i>per cent</i>
Kidneys	N I	6.97	3.45	17.28	1.26	0.85	18.08
	L 1	7.51	6.30	9.42	0.88	0.59	11.72
	2	6.68	4.67	8.21	1.10	0.74	16.47
	3	6.15	5.56	7.34	0.57	0.38	9.24
	4	6.01	5.05	6.90	0.50	0.34	8.37
	5	6.08	4.92	7.59	0.80	0.54	13.13
	D 1	8.02	6.77	10.04	1.22	0.82	15.21
	2	7.37	5.74	9.16	1.10	0.74	14.92
	3	7.17	6.32	8.30	0.51	0.34	7.11
	4	6.62	5.08	8.00	0.84	0.56	12.61
	5	6.58	5.68	7.80	0.57	0.38	8.63
	I 1	7.62	7.11	8.73	0.53	0.36	7.01
	2	7.34	5.60	8.94	0.99	0.67	13.46
	3	6.58	5.06	7.83	0.71	0.48	10.82
	4	6.43	5.48	7.78	0.78	0.53	12.19
	5	6.89	4.68	10.39	1.85	1.25	26.88
	O 1	7.88	6.16	10.24	1.14	0.77	14.44
	2	7.57	6.26	9.75	0.91	0.62	12.03
	3	7.48	5.68	9.99	1.14	0.77	15.24
	4	7.63	6.61	9.07	0.82	0.56	10.78
	M	8.08	6.37	10.44	1.04	0.70	12.86
Spleen	N I	0.531	0.035	1.714	0.214	0.144	40.30
	L 1	0.654	0.433	1.006	0.202	0.136	30.88
	2	0.618	0.294	1.080	0.258	0.174	41.75
	3	0.495	0.210	0.976	0.214	0.144	43.23
	4	0.462	0.197	0.890	0.182	0.123	39.39
	5	0.572	0.048	0.955	0.275	0.186	48.08
	D 1	0.768	0.314	1.334	0.299	0.202	38.95
	2	0.577	0.309	0.805	0.171	0.115	29.63
	3	0.706	0.375	1.353	0.266	0.179	37.67
	4	0.490	0.285	0.826	0.147	0.099	30.00
	5	0.637	0.295	1.239	0.289	0.195	45.36
	I 1	0.795	0.476	1.712	0.363	0.245	45.66
	2	0.669	0.323	1.442	0.293	0.198	43.80
	3	0.517	0.313	0.980	0.186	0.126	35.98
	4	0.504	0.286	0.857	0.148	0.100	29.45
	5	0.644	0.327	1.440	0.314	0.212	48.76
	O 1	0.697	0.509	1.095	0.167	0.113	23.96
	2	0.590	0.293	0.779	0.186	0.126	31.53
	3	0.800	0.295	1.988	0.457	0.308	57.13
	4	0.729	0.346	0.928	0.159	0.107	21.81
	M	0.680	0.270	1.250	0.274	0.185	40.26

TABLE II.

Results for Body and Relative Organ Weights According to Groups.

Organ	Group	Arithmetical mean	Minimum	Maximum	Standard deviation	Probable error	Coefficient of variation
		gm.	gm.	gm.	± gm.	± gm.	per cent
Heart	N I	2.85	1.95	4.42	0.35	0.24	12.32
	L 1	2.85	2.46	3.31	0.266	0.179	9.33
	2	2.91	2.52	3.57	0.307	0.207	15.50
	3	2.71	2.33	3.24	0.308	0.208	11.37
	4	2.49	2.16	2.74	0.177	0.119	7.11
	5	2.75	2.35	3.05	0.18	0.12	6.44
	D 1	3.09	2.55	3.64	0.32	0.21	10.26
	2	2.80	2.35	3.47	0.38	0.25	13.42
	3	2.66	2.52	2.85	0.11	0.07	4.14
	4	2.63	2.22	3.14	0.30	0.20	11.21
	5	2.76	2.31	3.09	0.23	0.16	8.40
	I 1	2.89	2.695	3.20	0.16	0.11	5.54
	2	2.96	2.44	4.36	0.504	0.34	17.03
	3	2.59	2.21	3.17	0.314	0.21	12.12
	4	2.81	2.54	3.21	0.21	0.14	7.44
	5	2.95	2.53	4.17	0.46	0.31	15.44
	O 1	3.14	2.77	3.78	0.28	0.19	9.17
	2	3.197	2.83	3.78	0.29	0.20	9.09
	3	3.09	2.62	3.84	0.36	0.24	11.62
	4	3.00	2.55	3.38	0.25	0.17	8.47
	M	2.98	2.15	3.46	0.32	0.21	10.59
Liver	N I	46.5	23.2	97.4	11.8	8.0	25.48
	L 1	55.8	46.5	73.0	6.88	4.64	12.33
	2	35.17	26.75	44.7	4.98	3.36	14.15
	3	40.45	31.2	51.1	5.81	3.92	14.35
	4	35.95	26.4	50.4	6.44	4.34	17.89
	5	35.35	27.8	43.4	4.25	2.87	12.02
	D 1	57.1	48.8	64.2	4.61	3.11	8.03
	2	49.1	34.7	61.4	7.99	5.39	16.26
	3	51.1	37.6	64.9	8.16	5.50	15.97
	4	52.2	36.1	76.6	11.82	7.97	22.64
	5	57.8	47.7	75.8	7.98	5.38	13.80
	I 1	46.1	38.5	55.3	4.70	3.17	10.20
	2	51.9	44.6	62.4	5.76	3.89	11.10
	3	37.4	29.9	42.5	4.18	2.82	11.18
	4	43.5	33.3	63.1	8.13	5.49	18.69
	5	46.0	32.7	64.9	11.17	7.53	24.31
	O 1	62.9	40.9	117.0	22.10	14.91	35.14
	2	46.6	33.1	82.6	13.70	9.24	29.40
	3	39.8	30.0	49.7	6.23	4.20	15.65
	4	47.6	31.4	72.5	12.10	8.16	25.27
	M	55.6	33.3	74.8	10.93	7.37	19.71

TABLE II—*Continued.*

Organ	Group	Arithmetical mean	Minimum	Maximum	Standard deviation	Probable error	Coefficient of variation
		<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	\pm <i>gm.</i>	\pm <i>gm.</i>	<i>per cent</i>
Brain	N I	5.11	3.33	8.16	0.87	0.58	16.92
	L 1	4.59	3.35	5.62	0.67	0.45	14.62
	2	4.82	3.77	6.48	0.92	0.62	19.07
	3	4.37	3.85	4.58	0.21	0.14	4.76
	4	4.26	3.70	5.12	0.42	0.29	9.95
	5	4.49	3.79	5.34	0.41	0.28	9.20
	D 1	5.49	4.30	6.58	0.69	0.47	12.56
	2	4.66	3.84	5.26	0.41	0.28	8.84
	3	4.77	4.06	5.49	0.47	0.31	9.74
	4	4.36	3.58	5.30	0.52	0.35	11.92
	5	4.77	3.91	5.68	0.63	0.43	13.22
	I 1	4.76	3.71	5.52	0.54	0.364	11.34
	2	4.83	4.39	5.44	0.39	0.264	8.10
	3	4.44	3.78	4.93	0.384	0.26	8.65
	4	4.21	3.67	4.81	0.375	0.25	8.91
	5	4.76	3.66	6.29	0.764	0.52	16.06
	O 1	5.49	4.33	6.52	0.70	0.47	12.71
	2	5.16	4.24	7.08	0.84	0.57	16.49
	3	5.18	3.94	6.52	0.94	0.64	18.17
	4	5.25	4.31	5.86	0.50	0.34	9.49
	M	5.41	4.73	6.70	0.51	0.34	9.31
Thyroid	N I	0.12324	0.0504	0.730	0.0757	0.05106	61.47
	L 1	0.09878	0.0661	0.1474	0.0256	0.01727	25.92
	2	0.09074	0.0650	0.1145	0.0172	0.01160	18.96
	3	0.09814	0.0722	0.1253	0.0133	0.00797	13.35
	4	0.12645	0.0620	0.191	0.0353	0.02380	27.92
	5	0.11624	0.0806	0.145	0.02514	0.01696	21.63
	D 1	0.10780	0.0656	0.1318	0.0271	0.0183	25.14
	2	0.11230	0.0720	0.1163	0.0256	0.0173	22.79
	3	0.12380	0.0987	0.1619	0.0201	0.0136	16.26
	4	0.11460	0.0726	0.128	0.0267	0.0181	23.30
	5	0.12680	0.0817	0.3351	0.0706	0.0476	55.68
		(0.10360)	(0.0817)	(0.1239)	(0.0140)	(0.0095)	(13.51)
	I 1	0.12270	0.0907	0.200	0.0316	0.0213	25.71
	2	0.13950	0.0862	0.201	0.0344	0.0232	24.65
	3	0.14800	0.0822	0.218	0.0342	0.0231	23.10
		(0.14027)	(0.0822)	(0.1822)	(0.0313)	(0.0211)	(22.31)
	4	0.18370	0.1010	0.392	0.0957	0.0645	52.07
		(0.1261)	(0.101)	(0.1585)	(0.0248)	(0.0167)	(19.69)
	5	0.14587	0.1108	0.201	0.0257	0.0173	17.68
	O 1	0.13110	0.0569	0.3211	0.0692	0.0467	52.80
		(0.10990)	(0.0569)	(0.1615)	(0.0294)	(0.0198)	(26.74)
	2	0.09960	0.0693	0.149	0.0238	0.0161	23.89

TABLE II—*Continued.*

Organ	Group	Arithmetical mean	Minimum	Maximum	Standard deviation	Probable error	Coefficient of variation
		<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>± gm.</i>	<i>± gm.</i>	<i>per cent</i>
Thymus	N I	1.230	0.334	2.720	0.451	0.304	36.64
	L 1	1.428	0.762	2.380	0.538	0.373	37.67
	2	1.187	0.604	2.120	0.399	0.269	33.61
	3	1.108	0.806	1.730	0.244	0.165	22.02
	4	1.268	0.959	1.540	0.241	0.163	19.01
	5	1.437	0.942	2.330	0.454	0.306	31.59
	D 1	1.415	0.632	1.900	0.360	0.243	25.43
	2	1.220	0.738	1.893	0.368	0.248	30.16
	3	1.142	0.435	1.480	0.337	0.227	29.50
	4	1.024	0.692	1.570	0.270	0.182	26.37
	5	1.116	0.524	1.790	0.391	0.264	35.03
	I 1	1.705	0.818	3.315	0.831	0.561	48.74
	2	1.364	0.602	1.960	0.390	0.263	28.60
	3	1.094	0.684	1.380	0.222	0.150	20.29
	4	1.117	0.315	1.720	0.394	0.266	35.25
	5	0.913	0.640	1.625	0.347	0.234	38.01
	O 1	0.977	0.525	1.930	0.487	0.329	49.85
	2	1.113	0.388	2.407	0.608	0.410	54.63
	3	1.137	0.471	1.722	0.402	0.271	35.36
	4	0.991	0.532	1.765	0.417	0.282	42.08
	M	1.420	0.483	2.370	0.601	0.405	42.29
Testicles	N I	2.61	0.470	4.93	0.729	0.492	27.93
	L 1	2.44	1.780	2.93	0.360	0.240	14.84
	2	2.77	1.950	3.54	0.530	0.360	19.03
	3	2.57	2.070	3.59	0.420	0.280	16.38
	4	2.51	1.880	3.01	0.420	0.280	16.61
	5	2.41	1.890	2.93	0.300	0.200	12.46
	D 1	2.82	1.960	3.53	0.540	0.360	19.14
	2	2.71	1.810	3.73	0.460	0.310	16.78
	3	2.90	2.330	3.81	0.440	0.300	15.27
	4	2.54	1.660	3.27	0.450	0.300	17.51
	5	2.37	1.550	3.10	0.500	0.340	21.01
	I 1	2.76	1.880	3.45	0.510	0.340	18.48
	2	2.72	2.100	3.49	0.470	0.320	17.35
	3	2.85	2.230	3.91	0.510	0.340	17.93
	4	2.63	1.770	3.39	0.460	0.310	17.60
	5	2.43	1.740	3.49	0.530	0.360	21.81
	O 1	3.29	1.160	4.22	0.980	0.660	29.64
	2	2.76	1.940	3.78	0.610	0.414	22.25
	3	2.63	1.160	3.82	0.850	0.570	32.28
	4	2.65	2.050	3.49	0.390	0.260	14.68
	M	2.59	1.810	3.56	0.490	0.330	18.87

TABLE II—*Continued.*

Organ	Group	Arithmetical mean	Minimum	Maximum	Standard deviation	Probable error	Coefficient of variation
		gm.	gm.	gm.	± gm.	± gm.	per cent
Hypophysis— Continued	O 1	0.01665	0.01223	0.02090	0.00256	0.00173	15.38
	2	0.01456	0.01115	0.01826	0.00185	0.00125	12.71
	3	0.01493	0.00994	0.01795	0.00255	0.00172	17.08
	4	0.01505	0.01095	0.02043	0.00278	0.00188	18.47
	M	0.01706	0.01239	0.02135	0.00227	0.00153	13.32
Suprarenals	N I	0.2088	0.0804	0.530	0.07503	0.05061	35.93
	L 1	0.1780	0.070	0.279	0.0526	0.03549	29.55
	2	0.2217	0.115	0.433	0.0846	0.05706	38.11
	3	0.2590	0.145	0.508	0.1187	0.08006	45.83
	4	0.2060	0.154	0.286	0.0403	0.02718	19.56
	5	0.2240	0.138	0.293	0.0496	0.03346	22.14
	D 1	0.1836	0.091	0.299	0.0543	0.0366	29.57
	2	0.2093	0.103	0.269	0.0576	0.0389	27.52
	3	0.1978	0.138	0.340	0.0680	0.0459	34.39
	4	0.2056	0.133	0.271	0.0442	0.0298	21.60
	5	0.2659	0.114	0.582	0.1317	0.0888	49.52
	I 1	0.1980	0.132	0.313	0.0479	0.0323	24.20
	2	0.2240	0.095	0.468	0.1038	0.0700	46.55
	3	0.1860	0.092	0.292	0.0592	0.0399	31.83
	4	0.2210	0.131	0.367	0.0690	0.0468	31.36
	5	0.2640	0.132	0.572	0.1330	0.0899	50.49
	O 1	0.2360	0.136	0.356	0.0644	0.0434	27.29
	2	0.2220	0.133	0.383	0.0836	0.0564	37.66
	3	0.2180	0.127	0.316	0.0549	0.0370	25.18
	4	0.1970	0.080	0.423	0.0983	0.0663	49.89
	M	0.1640	0.088	0.281	0.0575	0.0388	35.08
Pineal	N I	0.00885	0.00273	0.01873	0.00245	0.00165	27.68
	L 1	0.00654	0.00442	0.00899	0.00139	0.000938	21.25
	2	0.00623	0.00352	0.01035	0.00183	0.00123	29.38
	3	0.00643	0.00337	0.00890	0.00179	0.00121	27.84
	4	0.00602	0.00315	0.00834	0.00151	0.00102	25.11
	5	0.00653	0.00440	0.00777	0.00114	0.00077	17.46
	D 1	0.00832	0.00494	0.01415	0.00269	0.00181	32.33
	2	0.00643	0.00447	0.00836	0.00113	0.00076	17.57
	3	0.00829	0.00334	0.01173	0.00232	0.00156	27.92
	4	0.00737	0.00495	0.01236	0.00217	0.00146	29.44
	5	0.00744	0.00495	0.00930	0.00149	0.00101	20.02
	I 1	0.00755	0.00482	0.01004	0.00180	0.00121	23.86
	2	0.00824	0.00625	0.01012	0.00134	0.00090	16.26
	4	0.00678	0.00228	0.00851	0.00163	0.00110	24.04
	4	0.00672	0.00321	0.01045	0.00221	0.00149	32.83
	5	0.00806	0.00489	0.01050	0.00169	0.00114	20.97

TABLE II—*Continued.*

Organ	Group	Arithmetical mean	Minimum	Maximum	Standard deviation	Probable error	Coefficient of variation
		gm.	gm.	gm.	± gm.	± gm.	per cent
Thyroid— Continued	O 3	0.10030	0.0745	0.1825	0.0222	0.0150	21.71
	4	0.14950	0.0664	0.3135	0.0739	0.0499	49.51
		(0.13130)	(0.0664)	(0.227)	(0.0525)	(0.0354)	(39.97)
	M	0.16990	0.0615	1.230	0.2457	0.1657	144.65
		(0.11410)	(0.0615)	(0.2092)	(0.0343)	(0.0231)	(30.04)
Parathyroids	N I	0.00692	0.00109	0.02175	0.00278	0.00187	40.13
	L 1	0.00620	0.00353	0.00787	0.00093	0.00063	15.00
	2	0.00500	0.00284	0.00735	0.00115	0.00078	23.00
	3	0.00490	0.00268	0.00769	0.00141	0.00095	28.76
	4	0.00464	0.00203	0.00669	0.00123	0.00083	26.51
	5	0.00530	0.00356	0.00887	0.00151	0.00102	28.40
	D 1	0.00788	0.00431	0.01216	0.00263	0.00177	33.39
	2	0.00544	0.00252	0.00796	0.00154	0.00104	28.30
	3	0.00665	0.00428	0.00955	0.00161	0.00109	24.16
	4	0.00552	0.00296	0.00954	0.00221	0.00149	40.03
	5	0.00698	0.00240	0.01235	0.00341	0.00230	48.85
	I 1	0.00609	0.00482	0.01053	0.00164	0.00110	26.88
	2	0.00658	0.00384	0.01115	0.00210	0.00142	31.92
	3	0.00611	0.00355	0.01140	0.00202	0.00136	33.09
	4	0.00560	0.00251	0.00945	0.00207	0.00140	37.02
	5	0.00951	0.00559	0.01747	0.00351	0.00237	36.96
	O 1	0.00841	0.00426	0.01545	0.00309	0.00208	36.74
	2	0.00647	0.00346	0.01435	0.00279	0.00188	43.12
	3	0.00629	0.00266	0.00994	0.00163	0.00110	25.91
	4	0.00490	0.00178	0.00721	0.00154	0.00104	31.43
	M	0.00654	0.00346	0.01155	0.00215	0.00145	32.89
Hypophysis	N I	0.01540	0.00728	0.0294	0.00310	0.00209	20.27
	L 1	0.01308	0.00820	0.01735	0.00307	0.00207	23.47
	2	0.01434	0.01016	0.01745	0.00264	0.00178	18.42
	3	0.01254	0.01105	0.01445	0.00102	0.00069	8.10
	4	0.01210	0.01034	0.01372	0.00124	0.00084	10.25
	5	0.01156	0.00909	0.01410	0.00219	0.00148	18.95
	D 1	0.01578	0.01325	0.02048	0.00236	0.00159	14.95
	2	0.01401	0.01029	0.01858	0.00217	0.00146	15.48
	3	0.01475	0.01238	0.01856	0.00174	0.00117	11.79
	4	0.01121	0.00792	0.01516	0.00203	0.00137	18.10
	5	0.01385	0.00961	0.01745	0.00244	0.00165	17.61
	I 1	0.01400	0.01096	0.01654	0.00176	0.00119	11.93
	2	0.01595	0.01205	0.02005	0.00253	0.00171	15.87
	3	0.01330	0.01005	0.01770	0.00231	0.00156	17.37
	4	0.01352	0.00960	0.01750	0.00252	0.00170	18.65
	5	0.01460	0.01282	0.01960	0.00235	0.00159	16.10

TABLE II—*Continued.*

Organ	Group	Arithmetical mean	Minimum	Maximum	Standard deviation	Probable error	Coefficient of variation
		<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>± gm.</i>	<i>± gm.</i>	<i>per cent</i>
Axillary lymph nodes— Continued	O 1	0.072	0.050	0.088	0.0132	0.0089	18.33
	2	0.074	0.039	0.103	0.0180	0.0120	24.37
	3	0.112	0.050	0.237	0.0480	0.0330	43.04
	4	0.097	0.063	0.160	0.0290	0.0194	29.69
	M	0.102	0.036	0.240	0.0520	0.0350	51.02
Deep cervical lymph nodes	N I	0.08207	0.02052	0.211	0.03712	0.02504	45.23
	L 1	0.067	0.031	0.102	0.01830	0.01234	27.34
	2	0.066	0.033	0.099	0.02356	0.01589	35.70
	3	0.071	0.039	0.110	0.0231	0.01558	32.54
	4	0.051	0.033	0.087	0.0155	0.01046	30.39
	5	0.111	0.046	0.313	0.0879	0.05929	79.19
		(0.069)	(0.046)	(0.111)	(0.0214)	(0.0144)	(30.92)
	D 1	0.0957	0.062	0.147	0.0331	0.0223	34.56
	2	0.0926	0.038	0.182	0.0378	0.0255	40.81
	3	0.0758	0.046	0.199	0.0431	0.0291	56.83
	4	0.0568	0.022	0.138	0.0311	0.0210	54.75
	5	0.0758	0.044	0.236	0.0540	0.0365	71.28
		(0.0580)	(0.044)	(0.073)	(0.0090)	(0.0061)	(15.52)
	I 1	0.095	0.059	0.221	0.0452	0.0305	47.58
	2	0.078	0.051	0.096	0.0156	0.0105	20.00
	3	0.065	0.042	0.094	0.0155	0.0105	23.85
	4	0.068	0.046	0.136	0.0244	0.0165	35.86
		(0.061)	(0.046)	(0.0766)	(0.0098)	(0.0066)	(16.07)
	5	0.108	0.053	0.278	0.0656	0.0436	59.81
		(0.089)	(0.053)	(0.1681)	(0.0327)	(0.0220)	(36.66)
	O 1	0.085	0.059	0.132	0.0240	0.01620	28.26
	2	0.082	0.047	0.126	0.0214	0.0150	26.10
	3	0.093	0.034	0.143	0.0360	0.0240	42.90
	4	0.0904	0.046	0.227	0.0310	0.0210	34.46
	M	0.0902	0.032	0.311	0.0610	0.0410	67.65
		(0.0724)	(0.032)	(0.1449)	(0.0239)	(0.0161)	(32.93)
Mesenteric lymph nodes	N I	1.828	0.72	3.71	0.642	0.433	35.12
	L 1	1.543	0.868	2.068	0.352	0.237	22.81
	2	1.428	0.518	1.981	0.442	0.298	30.95
	3	1.530	0.955	2.560	0.421	0.284	27.52
	4	1.284	1.080	2.000	0.268	0.181	20.87
	5	1.347	0.810	2.280	0.389	0.262	28.88
	D 1	1.847	1.248	2.340	0.344	0.232	18.62
	2	1.468	0.886	1.974	0.313	0.211	21.32
	3	1.470	0.960	2.090	0.312	0.210	21.22
	4	1.292	1.066	1.650	0.163	0.110	12.61
	5	1.269	0.870	1.650	0.249	0.168	19.62

TABLE II—*Continued.*

Organ	Group	Arithmetical mean	Minimum	Maximum	Standard deviation	Probable error	Coefficient of variation
		<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	\pm <i>gm.</i>	\pm <i>gm.</i>	<i>per cent</i>
Pineal— Continued	O 1	0.00815	0.00469	0.01362	0.00299	0.00202	36.69
	2	0.00751	0.00588	0.01077	0.00163	0.00110	21.69
	3	0.00694	0.00263	0.01005	0.00228	0.00154	32.85
	4	0.00767	0.00456	0.01202	0.00265	0.00179	34.55
	M	0.00820	0.00307	0.01150	0.00212	0.00143	25.85
Popliteal lymph nodes	N I	0.13573	0.0525	0.382	0.04873	0.03287	35.90
	L 1	0.1320	0.096	0.193	0.0321	0.02165	24.32
	2	0.1200	0.084	0.201	0.0317	0.02139	26.42
	3	0.1230	0.082	0.163	0.0247	0.01666	20.08
	4	0.1110	0.074	0.177	0.0316	0.02131	28.47
	5	0.1100	0.068	0.138	0.0221	0.01491	20.09
	D 1	0.1583	0.123	0.228	0.0340	0.0229	21.48
	2	0.1471	0.117	0.201	0.0266	0.0179	18.08
	3	0.1149	0.056	0.146	0.0278	0.0188	24.18
	4	0.1187	0.077	0.149	0.0224	0.0151	18.86
	5	0.0988	0.061	0.149	0.0302	0.0203	30.54
	I 1	0.1570	0.105	0.208	0.0318	0.0225	20.25
	2	0.1310	0.085	0.196	0.0335	0.0226	25.57
	3	0.1190	0.079	0.192	0.0319	0.0215	26.81
	4	0.1160	0.078	0.147	0.0238	0.0161	20.53
	5	0.0950	0.052	0.177	0.0342	0.0231	36.19
	O 1	0.1060	0.075	0.153	0.0297	0.0200	28.02
	2	0.1370	0.072	0.196	0.0430	0.0296	31.31
	3	0.1530	0.085	0.231	0.0385	0.0260	25.16
	4	0.1380	0.078	0.248	0.0488	0.0329	35.36
	M	0.1490	0.077	0.283	0.0629	0.0424	42.20
Axillary lymph nodes	N I	0.09722	0.01865	0.2425	0.03779	0.02549	38.87
	L 1	0.084	0.059	0.125	0.02245	0.01514	26.72
	2	0.075	0.037	0.172	0.0397	0.02678	52.93
	3	0.078	0.054	0.142	0.0263	0.01774	33.72
	4	0.068	0.051	0.097	0.0146	0.00985	21.47
	5	0.065	0.041	0.088	0.0136	0.00917	20.92
	D 1	0.1059	0.078	0.188	0.0308	0.0208	28.28
	2	0.0817	0.035	0.120	0.0238	0.0161	29.14
	3	0.0848	0.037	0.111	0.0229	0.0155	26.99
	4	0.0716	0.036	0.098	0.0195	0.0132	27.21
	5	0.0652	0.044	0.077	0.0110	0.0074	16.88
	I 1	0.1010	0.050	0.140	0.0280	0.0189	27.75
	2	0.083	0.058	0.106	0.0166	0.0102	20.00
	3	0.083	0.050	0.114	0.0231	0.0156	27.83
	4	0.076	0.034	0.105	0.0222	0.0150	29.16
	5	0.063	0.041	0.091	0.0142	0.0096	22.57

TABLE II—*Concluded.*

Organ	Group	Arithmetical mean	Minimum	Maximum	Standard deviation	Probable error	Coefficient of variation
		<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>± gm.</i>	<i>± gm.</i>	<i>per cent</i>
Mesenteric lymph nodes— Continued	I 1	1.628	0.902	2.492	0.434	0.293	26.66
	2	1.549	0.883	1.890	0.302	0.204	19.43
	3	1.534	1.269	2.030	0.225	0.152	14.67
	4	1.417	0.980	2.430	0.392	0.264	27.67
	5	1.446	0.940	2.100	0.324	0.218	22.41
	O 1	2.376	1.105	3.430	0.857	0.578	27.65
	2	3.067	1.185	6.910	1.488	1.004	48.52
	3	2.340	1.640	3.270	0.539	0.364	23.03
	4	2.670	2.110	3.440	0.393	0.265	14.72
	M	2.220	1.450	2.880	0.389	0.262	17.51

ciated with these particular variations of light environment, it should be emphasized that other factors which might exercise an effect upon the mass relationships of organs will have to be considered before the full influence of the light factor can finally be estimated.

The evaluation of the results depends first, upon the significance to be attached to variations in weight of a given order with due regard for the occurrence of spontaneous variations of a similar order, and second, on the nature of the apparent effect produced. As has been mentioned, the experimental conditions employed involve but a few of the hypothetical factors that might affect organ weights. It was not possible to control all these, but an effort was made to equalize the influence of a number of conditions so that any effect they might have would be common to both the experimental animals and controls. The rabbits, of comparable breeds and age, were kept assembled for several weeks before the experiment in order to insure uniform periods of caging under the same environmental conditions. In addition, the same system of feeding was employed throughout the entire period of observation. Furthermore, a regular routine with reference to the time and order of postmortem examinations was followed, the experimental groups being killed on a certain day and the controls on the following day. The number of rabbits comprising each subgroup—10—was selected as being small enough to insure a satisfactory post-mortem examination, including the accurate weighing of organs and, at the same time, large enough to furnish data which could be used statistically. Finally, the comparatively short duration of the experiment tended to minimize the wide fluctuations of organ weights which occur in material examined over comparatively long intervals of time (3).

An idea of the uniformity of the various groups is obtained from the coefficients of variation (2). On the whole, the animal groups exposed to constant illumination or kept in the dark had the most uniform organ weights in the case of most of the organs, and next came the groups kept in an ordinary room (inside controls), while the rabbits which had recently been brought to the laboratory (outside controls) were relatively irregular in this respect. In the case of most organs, the coefficients of variation of the experimental groups are much smaller than those of the 350 normal rabbits previously studied (3), a fact which could hardly be accounted for upon the basis of the differ-

EFFECTS OF LIGHT ON NORMAL RABBITS, WITH ESPECIAL REFERENCE TO THE ORGANIC REACTION.

III. ANALYSIS OF ORGAN WEIGHTS.

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In Paper I of this series the purpose of the investigation was stated, and the experimental methods, the general clinical observations, and postmortem findings were described (1). The results of the experiment, in the form of organ weights, were presented in Paper II (2). In the present paper these results are discussed.

DISCUSSION.

It is evident that in considering the relationship between environment and the state of the animal organism, many factors are concerned, the effects and relative importance of which are difficult and perhaps impossible to estimate accurately. From a broad point of view, however, the state of the organism at any one time may be said to be conditioned by all the operative forces of physical environment together with the organism's response to their action. While, in the rabbit, variations in organ weight as well as more subtle functional changes, such as the body's reaction to certain disease processes, appear to be influenced by the amount and degree of change in the hours of sunshine (1), these cannot be the only environmental influences implicated. Other characteristics of sunlight and a number of totally different forces, some perhaps unsuspected as yet, undoubtedly participate in producing the alterations. In our experiment, the influence of light has been studied only under the following conditions: (1) constant illumination, (2) absence of shorter ultra-violet rays, and (3) continued exclusion of light. Although our present attention in this experiment is focused upon the alterations in organ weight asso-

tions on this point can be drawn. For the present, however, it seems advisable not to place too much stress on the results of statistical comparisons, or, in making such comparisons, to bear in mind the fact that, on the basis of comparisons which have been made, the differences found between the weights of comparable groups of normal animals are as a rule about $1/3$ to $1/2$ of the difference that might be expected. If this fact is taken into consideration in analyzing the results the differences in weight that are found are, in many instances, of considerable statistical significance.

However, if the usual basis of evaluation be followed, little statistical significance can be attached, in most instances, to differences in mean organ weight if a group to group comparison is made, but between the highest and lowest group values in a given series the difference is frequently sufficiently large to make it of probable significance. The controls resemble the two experimental series in these respects. With some organs, the ratios of the differences between extreme mean weights to the probable errors are larger with control than with experimental groups as might be expected in view of the spontaneous variations in organ weights of normal rabbits and, as will be seen, because of the general tendency toward organ weight uniformity shown by the groups exposed to constant light or to darkness. Furthermore, the differences that are found in comparing the mean organ weights of experimental groups with their appropriate controls or with so called normal values, as well as the differences found between experimental groups, vary from those of little or no statistical significance to those that have some statistical significance. In the majority of cases, however, the results of the statistical analysis have chiefly a negative value and the decision as to the significance of the findings has to be made on some other basis, such as the trend of a given curve or the direction of a possible change in weight of an experimental group of animals as compared with controls or with another experimental group. The application of this method of evaluation will become apparent as the results recorded in this investigation are discussed.

The present analysis of organ weights is primarily based upon a comparison with the mean organ weights from a group of 350 normal rabbits (3) because of the fact that the organ weights of the control

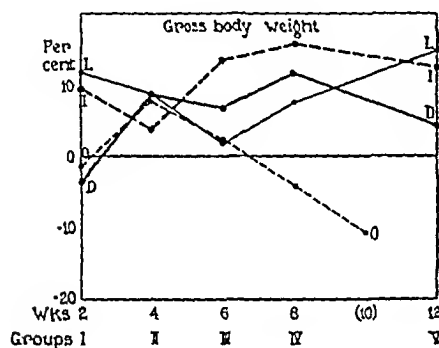
ences in the numbers of animals. However, the rabbits of the experimental series were a far more homogeneous animal stock than the large normal group.

From a statistical point of view, the significance to be attached to variations of organ weight of a given order is directly dependent upon the magnitude of the probable errors of these values. The probable errors in the case of our experiment were large and it would appear that little statistical significance could be attributed to the variations observed. However, in an analysis of the organ weights of two groups of normal rabbits, comprising 350 and 295 animals respectively, the probable errors of the mean weights of all the organs were extremely large, yet the average or mean weights of each organ were almost exactly the same in both groups (4). Similar conditions have been found to obtain with several groups of 10 normal rabbits killed on the same days and at a season of the year in which variation in organ weight was known to be marked. This closeness of agreement between mean organ weights would not be expected upon the basis of the magnitude of the probable errors, as shown by the following examples:

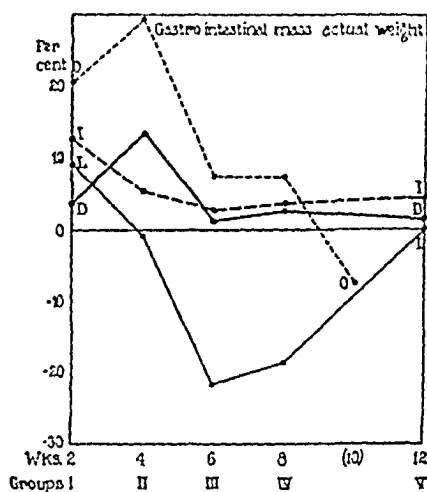
Organ		Mean weight	Probable error	Deviation P. E.
		gm.	gm.	
Liver.....	A	91.6	8.50	0.24
Actual.....	B	88.2	11.33	
Heart.....	A	5.02	0.78	0.0092
Actual.....	B	5.11	0.57	
Parathyroids.....	A	0.00653	0.00125	0.04
Relative.....	B	0.00655	0.00161	
Suprarenals.....	A	0.164	0.0376	0.0
Relative.....	B	0.164	0.0400	

These observations are highly significant and suggest that with material of this kind, the usual method of statistical evaluation based upon the ratio of the difference of mean weights to its probable error may not always be entirely satisfactory, although, as should be pointed out, additional data will have to be available before any general deduc-

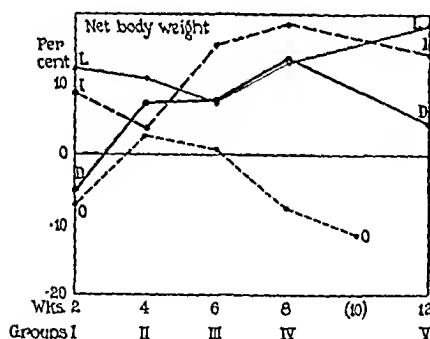
pronounced fluctuations, and its general level, moreover, is lower than those for the inside controls and for the dark series, while with Groups III and IV its position is considerably below the mean normal. The curve for the outside controls is the highest of the four; its general direction is downward, however, to a level approximating the mean normal. It will subsequently be seen that, in respect to the trend of weight variations, the gastrointestinal mass behaves in much the same



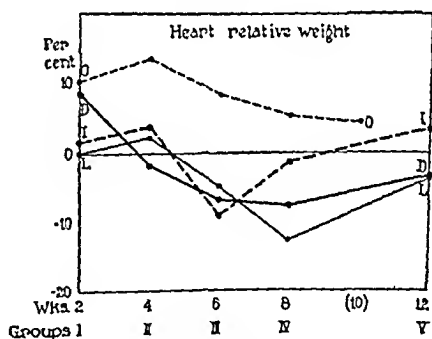
TEXT-FIG. 1.



TEXT-FIG. 3.



TEXT-FIG. 2.



TEXT-FIG. 4.

The curves in these and all other text-figures show the variations in mean weight as percentage deviation from the mean weights of 350 normal rabbits (3). The number of weeks refers, in each instance, to the time the groups of rabbits had been under conditions of constant light or of darkness, *i.e.* 2 weeks = Group I, 3 weeks = Group II, etc. The following system of representation is used in all text-figures:

Light groups ———	Inside controls - - - - -
Dark groups ———	Outside controls - - - - -

animals are not constant values but constitute a moving base. By the use of curves which have been plotted upon a basis of percentage deviation from these so called mean normal values, the general trend of organ weight obtaining in the control rabbits during the experiment is easily seen and the variations of the experimental groups are more readily appreciated. Only the curves for relative weights of organs have been used in the present discussion, except in the case of the gastrointestinal mass.

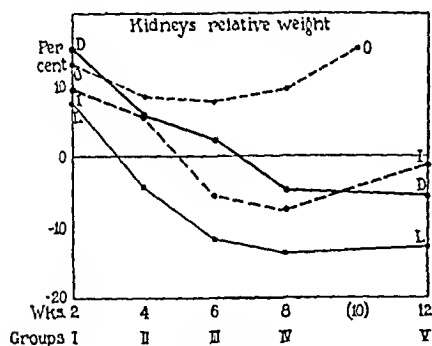
The organs have been considered in the following order: gross and net body weight, the gastrointestinal mass, the heart, the kidneys, the liver, the brain, the glands of internal secretion, the testicles, the spleen and thymus, and representative lymph nodes.

The terms, Groups I to V, used in this discussion refer to the groups of rabbits killed and examined 2, 4, 6, 8, and 12 weeks from the beginning of the experiment, these periods being the duration of exposure to conditions of constant illumination or of constant darkness. There is no Group V of outside controls but a series of normal rabbits, designated March normals, examined 10 weeks after the beginning of the experiment, is included.

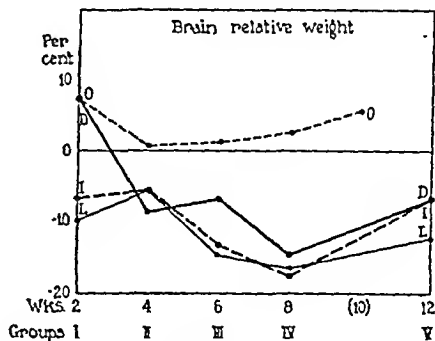
Gross and Net Body Weights.—The curves of gross and net body weights (Text-figs. 1 and 2) corresponding to the four animal series—light and dark groups, inside and outside controls—are identical in form, but the relative positions of the curves with respect to the mean value for the 350 normal rabbits are more accentuated in the case of the net body weights. All the curves of both gross and net body weights show irregularities, but those representing the successive groups of rabbits exposed to constant light are fairly regular and, in general, on an increasingly high level, well above the mean normal. The same is generally true of the curves for the inside controls. On the other hand, the curves for the dark room groups begin below the base line, and while they subsequently rise above it, their general level is below those of the light series and inside controls. The curves representing the weights of the outside control animals are generally below the others. Their low positions, corresponding to Group IV and the March normals, are influenced by the fact that at this time of year the available rabbit stock consists almost entirely of comparatively young animals.

Gastrointestinal Mass.—The curves illustrating variations in the actual weight of the gastrointestinal mass (including stomach, intestine, mesentery, omentum, and gastrointestinal contents) show definite differences in the case of the rabbits in the light environment (Text-fig. 3). The curve of the inside control groups is quite uniform and only slightly higher than the base line. With the exception of one irregularity (Group II) the same is true of the curve of the animals exposed to constant darkness. On the other hand, the curve of the light groups shows two

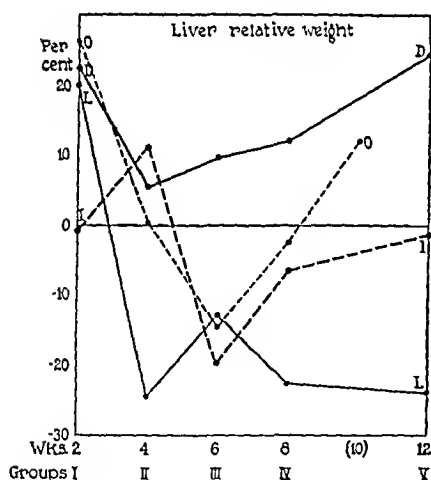
trols were all slightly higher than this value. The curve of the light series is, in general, the lowest, and that of the dark groups the highest, of the three representing animals caged within doors. The dark room curve is the most irregular and its fluctuations are the widest, while the light room curve is slightly but defi-



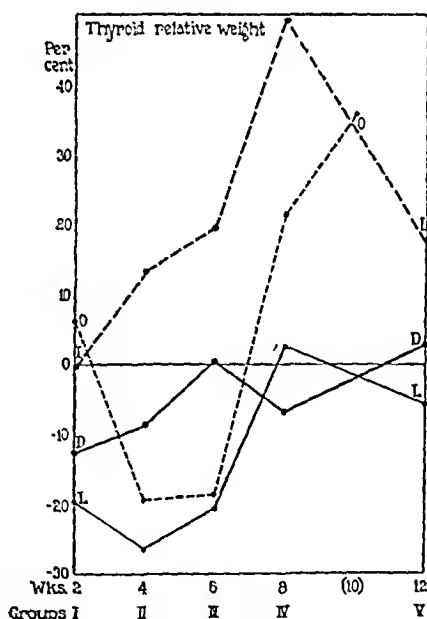
TEXT-FIG. 5.



TEXT-FIG. 7.



TEXT-FIG. 6.



TEXT-FIG. 8.

nately the most regular. It is of interest to note that, on the whole, the variations in relative brain weight, shown by the light, the dark, and the inside control groups, were more marked than those manifested by the heart.

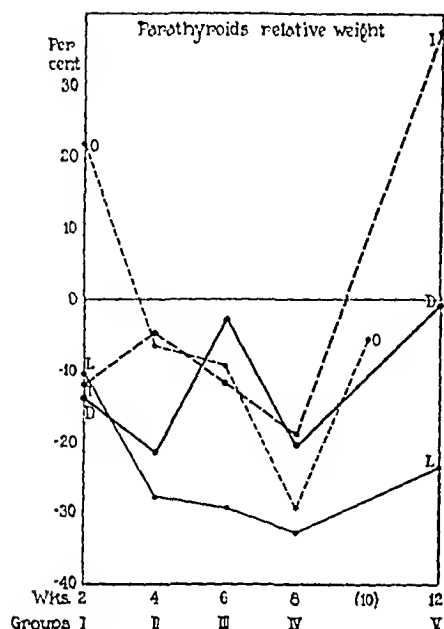
manner as the parenchymatous organs. This is particularly noticeable in the case of the light room animals.

Heart.—In the case of the heart, the curves illustrating the relative weights obtained from the dark and light series are, in general, on a slightly lower level than that for the inside controls (Text-fig. 4). In the first part of the experiment (Groups I, II, and III), all three curves are falling, but with Groups IV and V, the inside control curve rises fairly abruptly to above the mean normal, while the curves for both experimental groups do not rise until Group V and then do not reach this level. The curve for the outside controls is very uniform and well above the others.

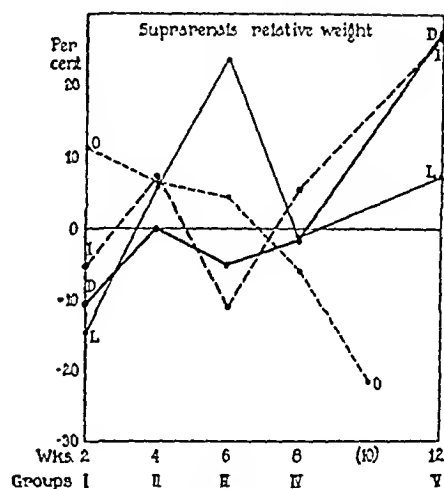
Kidneys.—The mean relative weights of the kidneys in rabbits under conditions of constant illumination progressively diminish in a remarkably uniform manner from a value slightly above the mean normal to one well below it. As is shown in Text-fig. 5, the curve for these groups is very regular and occupies the lowest position of the four. The curves for the dark and inside control groups also show a progressive but less pronounced and less regular decline from initial values somewhat above the mean normal, and there is a slight tendency for the values of the dark series to be larger than those of the inside controls. The curve for the outside controls is generally above the others, as it was in the case of the heart.

Liver.—The changes in the relative weights of the liver in association with conditions of constant light and of constant darkness were striking. During the time of this experiment, the relative liver weights of the inside control rabbits were in fair approximation to the mean normal but with a tendency toward smaller values (Text-fig. 6). The curve for the outside controls shows wider fluctuations, but there appeared to be a general tendency for the relative weights to be somewhat above the mean normal, although the value of Group III was considerably smaller than the others. In the case of the rabbits living under conditions of constant darkness, the weights of all groups were large and, on the whole, considerably above the mean normal. The curve begins at a high level and after one fall (Groups II) rises progressively to a point with Group V, which is approximately the same as that of Group I. On the other hand, the relative liver weights of the animals in an environment of constant light were, with the exception of Group I, extremely small. In both the first light and dark groups exposed to these conditions for 2 weeks, the relative weights of the liver were large and much larger than for the inside controls. With longer periods of exposure, however, the general tendency of relative liver weights was toward small values for the light and toward large values for the dark groups, respectively.

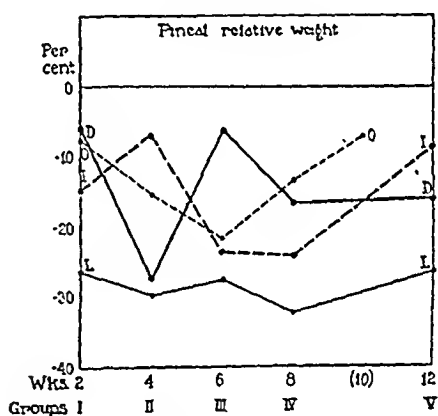
Brain.—In the case of the brain, the curves show that the relative weights of all the light and inside control groups were considerably below the mean normal, and with the exception of the first group, the same was true for the dark animals (Text-fig. 7). On the other hand, the relative weights of the outside con-



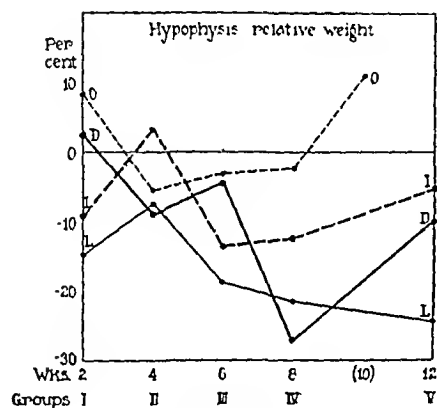
TEXT-FIG. 9.



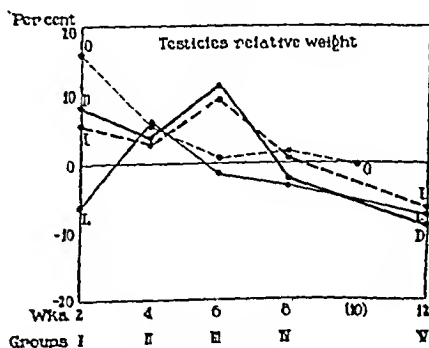
TEXT-FIG. 11.



TEXT-FIG. 12.



TEXT-FIG. 10.



TEXT-FIG. 13.

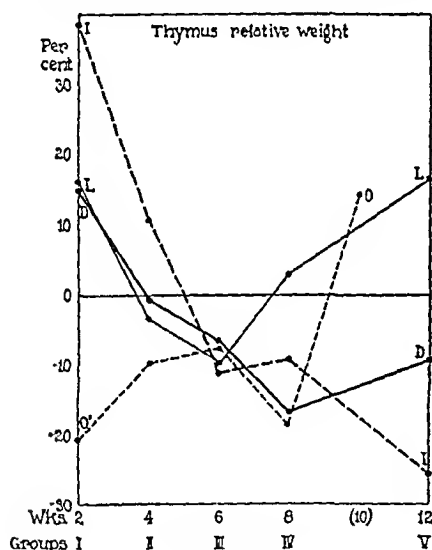
Thyroid.—The curve of relative thyroid weight for the inside control groups (Text-fig. 8) rises abruptly from a point which practically coincides with the mean normal value to a high level with Group IV, and then falls with Group V but to a value equal to that of Group III and higher than those of Groups I and II. There was a similar tendency among rabbits recently removed from outdoor conditions, although in two of the five observations (Groups II and III) the relative weights were very small. The curves for both light and dark groups differ in several respects from the control curves. Both start at lower levels, considerably below the mean normal, and in only three observations (Light IV, Dark III and V) do they reach this value. The general direction of the curve for the dark series is upward, and with the single fluctuation caused by Group III, is remarkably regular; its slope, however, is very gradual, and its final position is just above the mean normal. With the exception of Group IV, the curve for the light series is the lowest of the four. Its first portion (Groups I, II, and III) is far below the mean normal, and its rise with Groups IV and V, carries it only to this value. Although the curve for the rabbits in the constant light environment is not as uniform as that of the groups kept in constant darkness, the magnitude of its single fluctuation is much less pronounced than those of the inside and outside control curves, which swing first in one direction and then in another.

It would appear from these observations, that at a time when the trend of average relative weights of the thyroid of rabbits caged for some weeks in an ordinary animal room, or recently brought into the laboratory, was toward increasingly high levels, much above the mean normal, the general effects of caging for 2 to 12 weeks in environments of constant light or of constant darkness were, first, to restrain or depress this tendency to a marked degree, and, second, toward a stabilization of gland weight at a level below or approaching the mean normal value. In this connection it is interesting to note the group incidence of extremely large thyroids. Thus, there was one such gland in outside Groups I and IV and the March normals, and two in Group III and three in Group IV of the inside controls, while there was none in any of the light groups and only one among the dark room animals in Group V.

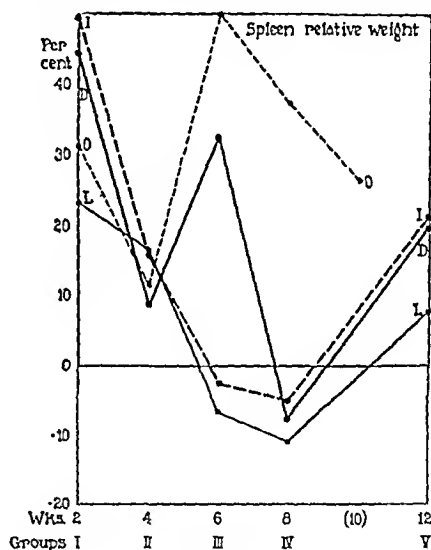
Parathyroids.—In regard to the parathyroid glands, constant illumination was again associated with diminished relative weights, as is clearly brought out in the curves (Text-fig. 9). During the first part of the experiment, the relative weights of the parathyroids of rabbits kept under unaltered conditions for many weeks (Inside Control Groups I, II, III, and IV) were small—from 5 to 15 per cent smaller than the mean normal—while the value of Group V was unusually high—37.5 per cent larger than the mean normal. The coefficients of variation of Groups IV and V were of the same order of magnitude, while that of Groups I, II, and III were slightly smaller (2). The curve for the outside controls begins considerably above the mean normal and ends just below it, but with the exception of its high initial position, its form is like that for the inside controls, while its level, corresponding to Groups II, III, and IV, is also in general agreement. The curve for the dark series is fairly comparable to that for the inside controls except

weight of the suprarenals at this time and that if a larger number of rabbits had been examined the average value might have been considerably lower, in which case the trend of relative suprarenal weights for the light series would have been toward a stabilization about the mean normal.

Pineal.—The outstanding feature of the observations on the pineal gland was the consistently low and very uniform relative weights of rabbits exposed to constant illumination. During the time of the experiment, the relative weights of both outside and inside controls (Text-fig. 12) were all lower than the mean normal and, in addition, were subject to rather wide fluctuations. The curve for the dark series shows equally pronounced weight variations in its first part but a tendency toward greater uniformity in its latter half (Groups IV and V). While the direction of this curve is not always the same as that for the inside controls, there is no



TEXT-FIG. 14.



TEXT-FIG. 15.

maintained trend which could be attributed with certainty to the effect of constant darkness. On the other hand, the relative weights of the pineal in the light groups were very small and uniformly so. It is remarkable that with an organ like the pineal, in which errors incident to removal and weighing must occur, the relative weights should be so consistently maintained in the successive light room groups that the curve is almost a straight line. Both the depressive and stabilizing effects on organ weight associated with the constant light environment of this experiment are well exemplified by the pineal gland.

Testicles.—In the case of the testicles (Text-fig. 13), there was practically no difference in the relative weights of the groups kept in the dark room and the inside controls, but there was a slight tendency toward smaller values on the part of the light series.

that the final rise corresponding to Group V is less marked. The differences in direction of the two curves, from Groups I to II and from Groups II to III may or may not be significant. The curve illustrating the light series is remarkably regular; it begins at the same general low level as those for the inside controls and dark groups and then falls continuously with Groups II, III, and IV to a much lower level which is only slightly increased with Group V. The terminations of the curves (Groups V) for the inside and outside controls and for the animals in the dark room are all considerably higher than the preceding observations (Groups IV).

Hypophysis.—With the hypophysis, the relative organ weights for the inside controls were, in general, smaller than the mean normal, while those for the outside controls were in fairly close approximation to it, as is shown by the curves in Text-fig. 10. The curve for the dark groups begins slightly above the mean normal (Group I), but with subsequent observations it falls considerably below this value and its general position is well below that for the inside controls. The curve illustrating the relative weights of the light series contrasts sharply with these curves both in respect to position and fluctuations. It occupies the lowest position and is by far the most regular, indicating again both a depressive and a stabilizing effect associated with the environment of constant light.

Suprarenals.—Variations in weight of the suprarenal glands of rabbits exposed to constant light or to constant darkness were less apparent than those of many other organs. In the case of recently acquired rabbits, the relative suprarenal weight (Text-fig. 11) steadily diminished from a value slightly above the normal mean as represented by Outside Control Group I to a value considerably below it (March normals). The first three observations on the inside controls showed slight fluctuations about the mean normal, but with Groups IV and V there was a well marked increase in relative weight and the curve rises sharply. Caging under conditions of constant darkness did not appear to have any particular effect upon relative suprarenal weights as compared with those of the inside controls, and except for a tendency toward uniformity of weight in the first four observations, the curve for these groups is quite similar to that for the inside controls. The results obtained from rabbits exposed to constant illumination are irregular and difficult of interpretation. The first two observations are similar to those of the inside controls and the dark series, but with Group III the curve rises sharply to a high point, then falls as abruptly with Group IV to the mean normal, and remains practically at this level with Group V. At the time of these last two observations, the relative weights of the suprarenals in the inside control groups were progressively increasing. The value of Light Group I is the lowest of the four as in the case of the majority of organs. It is possible that observations made earlier than the 2 week period would have shown still lower values and that the increasingly larger weights of Groups II and III represent conditions of readjustment with a subsequent stabilization at a level approximating the mean normal. On the other hand, one might assume, upon the basis of the very large coefficient of variation (2) that the high mean value for Group III was not fairly representative of the

for the inside controls with the exception of one pronounced upward fluctuation corresponding to Group III. In the case of the outside control groups the relative weights of the spleen were large, but the shape of the curve suggests a tendency toward decreasing weights which was interrupted by the high value of Group III. There was a marked irregularity in the weight of the spleen in the great majority of the groups as shown by the magnitude of the coefficients of variation (2), and in this respect these observations are in general accord with the two large series of normal rabbits (4). The high mean weights of Groups III of the dark and outside control rabbits were out of line with the prevailing tendency of decreasing weights, but the proportion of heavy organs in both groups indicates that their increased values were fairly representative although their coefficients of variation were of a greater magnitude than those of the preceding and succeeding groups. In this connection it should be mentioned that no pathological processes were found in the rabbits of either group which were considered sufficient to account for the larger size of the spleens. As far as Dark Group III is concerned, all that can be said is that it did not share in the general tendency toward decreased weight shown by other groups of the dark series or by the light and inside control groups during this period.

Lymph Nodes.—The last organs to be considered are representative lymph nodes: the popliteal, the posterior axillary, and the deep cervical nodes of both sides, and the main mesenteric mass of lymph nodes. Since similar changes in relative weights occurred in the two groups of superficial nodes, it seems sufficient to discuss but one of them. As will be seen in the curves of the posterior axillary nodes (Text-fig. 16), the relative weights of those of the inside controls decrease progressively from a point (Group I) close to the mean normal to one far below it (Group V). The curve for the outside controls begins (Groups I and II) well below the mean normal but rises with Groups III, IV, and the March normals to a general level slightly above this value. The curve for the dark series is very like that of the inside controls both in direction and position. The relative mean weights of the light groups, on the other hand, are all smaller than those of the dark room rabbits and the inside controls except for the last group the figure for which practically coincides with the final one for the others. In addition, the curve for the rabbits exposed to constant illumination is the most regular.

The trend of the weights of the deep cervical lymph nodes (Text-fig. 18) is in general conformity with that of the posterior axillary and popliteal nodes in the case of all four series of rabbits except for the increased values of Groups V of the inside controls and of the light and dark groups. There were a number of instances in which enlarged nodes were associated with a purulent inflammation of the nasal passages or cranial sinuses and others in which such a condition may have previously existed. In plotting the curves for the deep cervical nodes the weights of a few extremely large nodes in rabbits which showed an extensive purulent exudate in the cranial sinuses or nasal passages have been omitted from Groups V of the light, the dark, and the inside control series.

The results obtained with the main mass of mesenteric lymph nodes, as illus-

Thymic Mass.—The thymic mass apparently responded to the influence of constant illumination in a manner somewhat different from the organs already considered. As is seen from the curves (Text-fig. 14), the mean relative weights of the mass in the inside control groups progressively decreased from a high value of 40 per cent above the mean normal (Group I) to 26 per cent below it (Group V). During the same time, the observations for the four groups of outside controls were from 8 to 20 per cent below, while that of the March normals was 14 per cent above, the mean normal. This variation of the latter group from the general low level of the outside controls may, in part, be accounted for by the younger age of the rabbits and perhaps also by the fact that the majority of them had been in the laboratory for 4 weeks. It will be recalled that the inside control groups as well as those placed in the light and dark rooms had been assembled 4 to 6 weeks before the experiment was begun (1). It is possible that, other things being equal, conditions of indoor life are at first associated with a large relative weight of the thymic mass. The curve of relative thymus weights for the dark series is similar to that of the inside controls except that it begins at a lower level and that it rises slightly at the end. While such minor differences may not be of any significance, they suggest a tendency toward stabilization of relative organ weight not observed in the inside control rabbits. In regard to the rabbits exposed to constant illumination, the first part of the curve, corresponding to the first three groups, is almost identical with the curve for the dark room animals. From this point on, however, the curve rises abruptly with Group IV, and continues to rise with Group V to a value practically identical with that of Group I, that is, 17 per cent above the mean normal. At this time, the relative thymus weights of the inside controls were progressively diminishing and the value for Inside Group V was 26 per cent below the mean normal.

In connection with these results, the question arises as to whether well marked differences in relative weights of the thymic mass might not be accounted for on the basis of variations in fat content rather than in actual thymic tissue. However, in the case of Groups V of the light room rabbits and the inside controls, there was little difference in gross and net body weights or in the general state of body fat at autopsy, so that an increase in the weight of the thymic mass due to an accumulation of fat might be as readily assumed in one group as in the other.

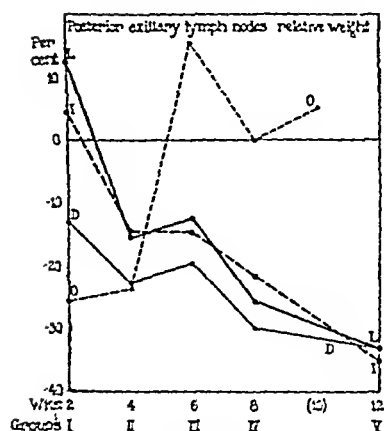
Spleen.—While the results obtained with the spleen do not indicate any striking weight variations which can be associated with the experimental light and dark conditions, the observations of the light groups suggest a slight effect. At the beginning of the experiment, the mean relative weight of the spleen was large in all groups (Text-fig. 15), but that of the light group was much the smallest of the four. From the high initial value, there was a progressive decrease in relative weights of the inside control groups (Nos. III, IV, and V), reaching a value slightly below the mean normal but with Group V somewhat exceeding it. The curve for the light series is similar in form to that of the inside controls, but its general position is somewhat lower and it is more regular. With the dark groups there was a similar general trend toward smaller relative weights. The curve is very like that

trated in the curves (Text-fig. 19), show that the relative weights in the case of the animal groups recently brought to the laboratory were much greater than the mean normal and, in addition, were quite irregular, as the marked fluctuations of the curve illustrate. On the other hand, the relative mean weights for the inside controls were somewhat smaller than the mean normal with a slight tendency toward decreasing values; their comparative regularity is shown by the smoothness of the curve. The curves for both experimental groups closely resemble that for the inside controls. With the exception of the first dark group, both curves are slightly but consistently lower, but the difference is a minor one and little significance can be attached to it.

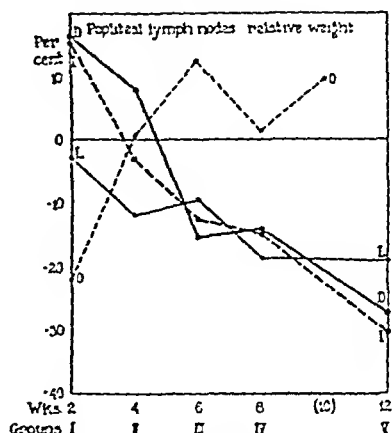
These results may be summarized as follows: With an environment of constant illumination two principal effects upon organ weight were observed. There was a definite tendency toward small relative weights which was particularly striking in the case of the liver, the kidneys, the gastrointestinal mass, the thyroid, the parathyroids, the hypophysis, the pineal, the spleen, and representative lymph nodes; it was less pronounced with the heart, the brain, and the testicles. Moreover, with some organs such as the liver, the thyroid, and the parathyroids, this tendency could be noted when the organs of rabbits living under ordinary indoor conditions were large or were increasing in weight. The thymus appeared to be an exception to this general effect of diminished organ weights, for the last two observations showed increasingly large values, while those of the controls continued to be progressively smaller.

The second effect associated with conditions of constant illumination may be described as a stabilization of organ weight in contrast with the more pronounced irregularities or fluctuations observed in the control groups. The curves illustrating the mean relative organ weights of the groups exposed to the light environment are in most instances more regular than those of the controls, while the smaller coefficients of variation in the majority of the light room organs indicate the comparative weight uniformity within the groups (1).

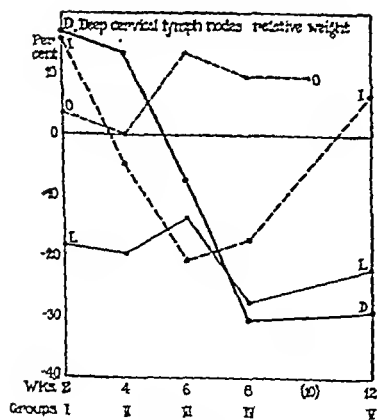
The effects upon organ weights were less evident in the case of rabbits living under conditions of light exclusion for the same periods of 2 to 12 weeks. A tendency toward smaller mean weights as compared with the control values was observed in the case of the relative weights of the heart, the parathyroids, the hypophysis, the pineal, and the thymus, and to a considerably greater degree with the thyroid. A



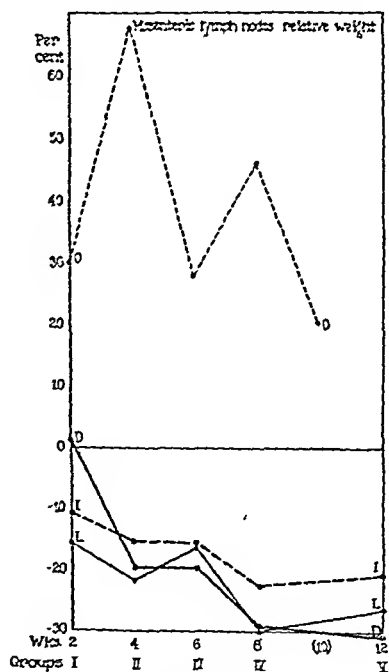
TEXT-FIG. 16.



TEXT-FIG. 17.



TEXT-FIG. 18.



TEXT-FIG. 19.

light groups and the lymph nodes of the two experimental and the inside control series. It would appear that the causative factor or factors responsible for this type of variation in organ weight were operative throughout the period of observation, that these particular organs were capable of a continued response in a like manner during this time, and that, in addition, no force capable of eliciting a response in a different direction was effective in changing the organ weight reaction. The third type of curve resembles the first except that there is a tendency for it to begin on an approximately horizontal level, as illustrated by the curves for the thyroid of the light and dark series and for the parathyroids and suprarenals of the inside controls and the dark groups. The shape of these curves suggests an initial response to some cause which either ceased to be effective in this respect or was no longer operative, or else that another causative factor acting in the opposite direction superseded or effectively opposed the first. These examples indicate the complex nature of the relationship between physical environment on the one hand and the animal organism on the other. But, with due regard for other factors which may enter into this relationship, the general types of the curves obtained in this experiment are corroborative evidence of a weight reaction on the part of the organs of the body in response to the influence of external forces as represented by physical environment.

The observations recorded in this study bring out the significant fact that the variations in organ weight associated with the artificial environments employed all fell within the so called normal limits as determined by a study of 645 stock rabbits (4). From the point of view of organic reaction, therefore, our experimental conditions must be considered as being within the scope of action of the physical factors which ordinarily contribute to the occurrence of spontaneous variations of organ weight. It is noteworthy, however, that the variations which did occur tended to approach the extreme limits of spontaneous variation and to persist there.

An interesting feature of this experiment was the character of organ weights of the groups of rabbits kept in an unaltered room as contrasted with those recently brought to the laboratory. In general, the weights of organs per kilo of body weight were somewhat smaller and more uniform in the indoor controls, and with certain organs,

tendency toward uniformity or stabilization of organ weight was also observed with some organs, especially the thyroid and the heart. But the most striking feature of the results obtained in this series of animals was the marked and sustained high weight values for the liver. Both the actual and relative weights were very large and were considerably greater than those of the controls. This effect upon the liver was not shared by the other parenchymatous organs, and while no reason for its occurrence can be stated with certainty, it may possibly be associated with an adjustment of metabolic activity. Mention was made in Paper I (1) that some physical deterioration in the rabbits kept in constant darkness was suggested by the body weight curves and the general state of body fat of certain animals at autopsy. The large size of the liver in the groups of dark room rabbits may be significant in this connection.

The lymph nodes as a class showed practically no weight alterations in the rabbits caged under conditions of constant darkness, and only slightly smaller values in the animals kept under constant illumination, other than those which also occurred in the control rabbits living in an unaltered animal room.

The curves illustrating the variations in mean organ weight are of three principal types which, in conjunction with the experimental conditions and the variability of organ weight known to occur spontaneously, are strongly suggestive of a causal relationship between the external factors of physical environment and the mass relationships of organs. The most common type of curve has an initial downward movement with a final upward swing, as is illustrated by the graphs of the gastrointestinal mass, the thymus, and the parathyroids of the animal groups exposed to constant illumination and those of the heart and the liver of the rabbits living in constant darkness. The shape of these curves suggests first, a primary reaction to some force or stimulus followed by a failure to maintain this response, and second, the occurrence of a reaction in an opposite direction which, it may be conjectured, was due either to a simple readjustment or to the operation of a dissimilar stimulus. A second type of curve is characterized by a progressive downward trend with little or no tendency toward a final reversal of this movement, as is shown by the results obtained with such organs as the liver, the kidneys, and the hypophysis of the

unlikely that as our knowledge of the diverse factors which affect the mass and mass relationships of organs increases, some will be found manifesting effects comparable with those observed under the conditions of this experiment, while others will be associated with dissimilar effects. The results of any experiment of this type, therefore, will depend upon all the factors involved and the extent or degree to which their influence upon organ weight is manifested.

SUMMARY AND CONCLUSIONS.

An analysis has been made of the organ weights of normal rabbits exposed to a constant illumination having none of the shorter ultraviolet rays and of other rabbits kept in darkness for periods of 2 to 12 weeks.

The environment of constant illumination was associated with a well marked decrease in the relative weights of most organs, and in certain instances this occurred when the organ weights of the controls were becoming increasingly large. There was also an associated effect of stabilization of organ weight.

The majority of the organs of rabbits caged in constant darkness also showed a tendency toward decreased and stabilized weights, but these effects were less pronounced than in the rabbits caged under conditions of constant illumination. A notable exception to this general result was the weight of the liver which was markedly increased.

The results of this experiment support the conception that there is a relationship between light and the physical state of the animal organism which may be expressed in the concrete form implied by the trend or direction of organ weight.

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3. Brown, W. H., Pearce, L., and Van Allen, C. M., *J. Exp. Med.*, 1925, xlii, 69.
4. Brown, W. H., Pearce, L., and Van Allen, C. M., *J. Exp. Med.*, 1926, xliii, 733.

the thymus and the lymph nodes in particular, this tendency appeared to be more marked with the longer periods of the experiment. A sedentary cage life with a regular and abundant food supply might be expected to affect the weights of certain organs and perhaps particularly those more immediately concerned with metabolic activities. The most pronounced weight diminution occurred in the main mass of mesenteric lymph nodes, but the superficial lymph nodes also showed almost as striking an effect. However, there were definite fluctuations of mean weight of other organs in the inside control series, and in the case of the thyroid, the parathyroids, and the liver, for example, pronounced variations were observed. While there is no doubt that conditions of indoor life may affect organ weight of rabbits, other influences such as age and breed must be considered before the relative importance of this factor can be estimated.¹

Little is known either of the weight relationships of one organ to another in normal or diseased states or of the factors that may affect these relationships. It seems unwise, therefore, to carry the analysis of results further at the present time.

Finally, it should be pointed out that while these experimental results indicate that an environment of constant light having none of the shorter ultra-violet rays and one of practically constant exclusion of light were associated with and presumably were the cause of definite variations in the physical state of certain organs of normal rabbits, it cannot be assumed that similar effects, either of degree or even of kind, would necessarily be obtained even with the use of the same environments. A different state or condition of organs at the initiation of experimental procedures might operate toward the production of different results, while the effect of various forces during the experimental period cannot be predicted. It is not

¹ In connection with the effect of indoor cage life upon organ weight, it is of interest to note that the reaction to such an experimental disease as a transplantable malignant neoplasm is not necessarily the same in rabbits caged indoors for comparatively long periods and in rabbits which have been in the laboratory for only a few days prior to inoculation. In an experiment carried out in February and March, 1926, the malignancy of the tumor in recently acquired rabbits was considerably greater than in those animals which had been in the laboratory for several weeks.

tion and multiplication.^{4,5} The products of tryptic digestion and complete peptic digestion of embryo juice protein were also unable to support cell proliferation, under the conditions of our experiments.

Further investigation of the processes of cell nutrition led to unexpected results: tissue cells were found to utilize the higher cleavage products of the protein molecule for multiplication.^{6,7} The purpose of the present paper is to report the experiments in the course of which this important fact was discovered.

Technique for Preparing the Protein Split Products.

The protein split products were obtained by digesting embryonic pulp, egg white, rabbit brain, and commercial fibrin presumably from ox blood, at 40°C. with Fairchild's pepsin in N/20 HCl. The process was carried out for different lengths of time, and the ratios of pepsin to protein and of protein and pepsin to acid were varied in such a manner as to obtain products representing different states of digestion. The degree of digestion was followed by amino nitrogen determinations. Afterwards, the different solutions were brought to a pH of 7.0 by the addition of normal NaOH, and boiled down to somewhat less than half their original volume, in order to destroy the pepsin and remove the toluene which was added in some instances as an antiseptic. The boiling was also designed to remove any unchanged protein or heat-precipitable products and to concentrate the salts. After determination of the cryoscopic point, the solutions were rendered isotonic by the addition of water or Ringer solution of five times its usual concentration. The pH was adjusted at 7.2 to 7.4, and the ratio of total nitrogen to amino nitrogen determined.

Split products were also obtained by autoclaving the protein in water, in N/10 HCl or NaOH in a steam sterilizer at various pressures for different lengths of time. The pH was then adjusted and the solution prepared as described above.

Technique for Testing the Growth-Promoting Action of the Protein Cleavage Products.

The growth-promoting action of the above substances at several concentrations was tested on fibroblasts, according to two techniques which have already been described. In the first procedure,⁸ fragments of heart were cultivated on

⁴ Carrel, A., and Ebeling, A. H., *Compt. rend. Soc. biol.*, 1924, xc, 31.

⁵ Baker, L. E., and Carrel, A., *J. Exp. Med.*, 1926, xlv, 397.

⁶ Carrel, A., *Compt. rend. Soc. biol.*, 1926, xciv, 1060.

⁷ Carrel, A., and Baker, L. E., *Proc. Soc. Exp. Biol. and Med.*, 1926, xxiii, 627.

⁸ Ebeling, A. H., *J. Exp. Med.*, 1921, xxxiv, 231.

THE CHEMICAL NATURE OF SUBSTANCES REQUIRED FOR CELL MULTIPLICATION.

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It is well known that tissue cells live indefinitely when cultivated in plasma and embryonic juice.¹ In such a medium, strains of fibroblasts and epithelium have proliferated without interruption for several years. It appeared that the chemical substances from which the cells synthesize new protoplasm are constituents of embryo juice and not of blood serum.² However, it has not been ascertained whether the building up of new cells merely requires nutrient materials present in the embryo juice or whether the juice also contains some specific hormone essential to cell division. Many attempts have been made to determine the nature of the substances used by the tissues, and to isolate the principle responsible for growth. Embryonic tissue extracts were fractionated and the activity of each fraction was tested on fibroblasts. These experiments pointed out that the growth-promoting substance consisted of a protein or was closely associated with a protein.³ The suggestion was made that the nitrogenous material required by the tissues might be a product obtained from the protein by the cells through some ferment action.³ It was also found that amino acids and other dialyzable nitrogenous compounds of the embryonic juice caused no increase in the mass of the tissues, although they had the property of stimulating cell migra-

¹ Carrel, A., *J. Exp. Med.*, 1912, xv, 516; 1913, xvii, 14. Ebeling, A. H., *J. Exp. Med.*, 1922, xxxv, 755; *Compt. rend. Soc. biol.*, 1924, xc, 562; *J. Exp. Med.*, 1925, xli, 337.

² Carrel, A., and Ebeling, A. H., *J. Exp. Med.*, 1921, xxxiv, 317; 1923, xxxvii, 759.

³ Baker, L. E., and Carrel, A., *J. Exp. Med.*, 1926, xlv, 387.

varying from 1 to 2 weeks. The cultures were placed under the projectoscope daily, and the area of the colonies was traced and measured with the planimeter. The action of the experimental substances was expressed by a graph on which the area of the colonies was plotted as a function of the time.

Action on Fibroblasts of Embryo Tissue Hydrolyzed by Pepsin.

Embryonic tissues reduced to a fine pulp in a Latapie apparatus were digested with pepsin for 32, 16, and 3.5 hours respectively, and a comparison was made of the effects of these products and of Ringer solution on fibroblasts migrating from heart tissue. The products of 32 and 16 hours digestion showed no growth-stimulating action (Table I), and appeared to be somewhat toxic, the cells generally becoming loaded with fat granules and losing their activity. On the contrary, those obtained by 3.5 hours digestion showed a decided stimulating action, the width of the new growth being two and occasionally three times as great as that of the control in Ringer solution (Table I). The ratio of total to amino nitrogen was determined in each of these digests. The 32 and 16 hour digests had a ratio of 2.25 and 2.21 respectively, while the ratios in two different 3.5 hour digests were 4.34 and 6.7. The first two were slightly toxic, while the last ones promoted tissue growth, the greater growth being observed in the last digest. This indicated that the tissues utilized certain intermediate cleavage products for growth, probably some of the larger fragments of the protein molecule.

Action on Fibroblasts of Egg White Hydrolyzed by Pepsin.

The products of partial peptic hydrolysis of egg white were also tested on fibroblasts. The length of the time of digestion varied, but the protein was never completely hydrolyzed and some of the larger cleavage products remained in solution. These substances always determined a large growth of fibroblasts (Table II). It was thus obvious that nutrient substances could be obtained from egg white by pepsin hydrolysis.

Action on Fibroblasts of the Cleavage Products of Commercial Fibrin.

A similar effect on fibroblasts was exhibited by the cleavage products of a protein unrelated to that of chicken embryo or egg white, namely,

hollow slides in a control medium composed of equal parts of plasma and Ringer solution, and in an experimental medium composed of equal parts of plasma and the protein cleavage products. Two or three cultures were made in each of several different concentrations of the experimental material, and allowed to grow for 48 or 72 hours. The growth was measured by the width of the ring of new tissue, or by the increase in area of the colonies. In the second procedure,⁹ heart tissue or fibroblasts were cultivated in flasks containing 0.5 cc. serum, 1 cc. Tyrode

TABLE I.

Rates of Growth of Fibroblasts in the Peptic Digestion Products of Embryo Tissue.

Culture group No.*	Chemical preparation No.	Digestion	Total nitrogen	Amino nitrogen	Total N Ratio: Amino N	Ringer solution	Width† of new tissue				Time of growth	Ratio: Peptic digest Ringer solution			
							Peptic digest					Pure	Diluted		
							Pure	Diluted							
								1:2	1:4	1:8					
		hrs.	per cent	per cent						hrs.	Pure	1:2	1:4	1:8	
3171 A	X-274	32	.378	.168	2.25	1.5	2.0	1.5	2.0	48	1.3	1.0	1.3		
8649 D†	X-271	16	.298	.135	2.21	5.0				4.0	48				.8
8643 D	X-271	16	.298	.135	2.21	2.0	2.0	2.0	2.0	48	1.0	1.0	1.0		
3178 A§	X-276	3.5	.352	.0525	6.7	1.5	5.0-6.0		4.0	5.0	48	3.6		2.6	3.3
3185 A§	X-276	3.5	.352	.0525	6.7	3.0		7.0	9.0	6.0	72	2.3		3.0	2.0
3192 A	X-276	3.5	.352	.0525	6.7	2.0	2.0	4.0	3.0	48	1.0		2.0	1.5	
3201 A	X-287	3.5	.358	.0825	4.34	1.5	6.0	2.5	2.0	48	4.0		1.7	1.3	
3219 A	X-287	3.5	.358	.0825	4.34	3.0	7.0	3.0	2.5	48	2.3		1.0	.83	

*Each culture group number represents 6 to 10 experiments.

† Measured under the microscope in 0.1 mm. units.

‡ Experiments were also made at dilutions of 1:12 and 1:16. These showed no increase over the control.

§ Tyrode solution was used as control in place of Ringer solution.

|| A second control solution, containing pepsin treated in the same way as the experiment, was used. The cultures in the control pepsin solution showed less growth than those in the control Ringer solution.

solution, and 0.5 cc. embryonic tissue extract. After coagulation, 1 cc. of Tyrode solution was added to the controls and 1 cc. of the preparation to the experiments. Every 2 days, the fluid medium was removed, the coagulum washed with 2 cc. Tyrode solution, and fresh fluid added. When digestion of the fibrin occurred, the coagulum of both control and experiment was patched with 0.25 cc. plasma and 0.25 cc. embryonic extract. The tissues were allowed to grow for periods

⁹ Carrel, A., *J. Exp. Med.*, 1923, xxxviii, 407.

some cases, the volume of the tissue became four times as large as that of the controls (Fig. 3). Colonies of fibroblasts 2 cm. in diameter were obtained.

TABLE III.

Rate of Growth of Fibroblasts in the Peptic Digestion Products of Commercial Fibrin.

Culture group No.*	Chemical preparation No.	Width† of new tissue				Time of growth	Ratio: $\frac{\text{Peptic digest}}{\text{Ringer solution}}$		
		Ringer solution	Peptic digest				Pure	Diluted	
			Pure	Diluted					
				1:2	1:4				
						hrs.			
8682 D	X-297	3.0	6.0	10.0	12.0	72	2.0	3.3	4.0
8693 D	X-297	2.5	6.0	5.0	6.0	48	2.4	2.0	2.4
3232 A	X-297	3.0	5.0	5.0		48	1.7	1.7	
3225 A	X-297	2.0	5.0	3.0	3.0	48	2.5	1.5	1.5
8683 D	X-298	2.0	3.0	3.0	3.0	48	1.5	1.5	1.5
8692 D	X-298	2.0	4.0	6.0	6.0	48	2.0	3.0	3.0
8703 D	X-298	2.5		5.0		48		2.0	
	X-299	2.5		6.0		48		2.4	
3233 A	X-298	2.0	4.5	3.5		48	2.25	1.75	
8703 D	X-299	4.0		6.0		48		1.5	
3234 A	X-299	4.0	5.0	5.0	6.0	48	1.25	1.25	1.5
8696 D	X-303	4.0	7.0	8.0	5.0	48	1.75	2.0	1.25
3288 A	X-329	2.0	6.0			48	3.0		
8710 D	X-310	2.0	3.0	3.5	3.0	48	1.5	1.75	1.5
3265 A	X-318	1.5	4.0			48	2.6		
	X-302		4.0			48	2.6		
	X-304		4.0			48	2.6		
3277 A†	X-324	2.5	4.5			48	1.8		
	X-325		6.0			48	2.4		
	X-326		6.0			48	2.4		
8732 D‡	X-324	1.0	1.5			48	1.5		
	X-325		4.5			48	4.5		
	X-326		6.0			48	6.0		

* Each culture group number represents 8 experiments.

† Measured under the microscope in 0.1 mm. units.

‡ Preparations X-324, X-325, and X-326 varied considerably in the degree of hydrolysis. The ratio of total to amino nitrogen was 5.2, 6.3, and 9.0 respectively.

In order to secure some information concerning the nature of protein cleavage products utilized by the tissues, three different digests were prepared by varying the ratio of the weight of fibrin to the weight

commercial fibrin (Table III). The growth-promoting action of some of the hydrolyzed products of fibrin was even greater than that of the embryonic tissue and egg white digests. This phenomenon may be due merely to the state of digestion reached by the protein, rather than to its nature. The split products of fibrin not only brought about

TABLE II.

Rate of Growth of Fibroblasts in the Peptic Digestion Products of Egg White.

Culture group No.*	Chemical preparation No.	Width† of new tissue					Time of growth hrs.	Ratio: $\frac{\text{Peptic digest}}{\text{Ringer solution}}$				
		Ringer solution	Peptic digest					Pure	Diluted			
			Pure	Diluted					1:2	1:4	1:8	
				1:2	1:4	1:8						
8667 D	X-288	2.3	2.5-4.0	4.0	2.5		48	1.1-1.7	1.7	1.1		
8673 D	X-282	3.0	5.0	4.0	3.0		48	1.7	1.3	1.0		
8680 D	X-288	1.5	2.0				48	1.3				
	X-288 a	1.5	3.0				48	2.0				
	X-291	1.5	3.5				48	2.3				
	X-291 a	1.5	2.5				48	1.7				
	X-305	3.0	6.0	4.0	3.0		48	2.0	1.3	1.0		
8702 D	X-305	3.0	6.0	4.0	3.0		48	2.0	1.3	1.0		
3177 A‡	X-275	1.5	4.0		4.0	3.0	48	2.6		2.6	2.0	
3184 A‡	X-275	3.0	11.0		10.0	9.0	72	3.6		3.3	3.0	
3191 A‡	X-275	2.0	5.0		3.0	3.0	48	2.5		1.5	1.5	
3196 A	X-282	1.5	2.5	2.5	2.0		48	1.6	1.6	1.3		
8686 D	X-288	2.5	2.5				48	1.0				
	X-291	2.5	5.0				48	2.0				
	X-292	2.5	6.0				48	2.4				
	X-282	2.5	6.0				48	2.4				
	X-291	4.0	8.0		9.0	5.0	72	2.0		2.2	1.2	
3209 A	X-291	4.0	8.0		9.0	5.0	72	2.0		2.2	1.2	
3212 A	X-292	2.0	4.0	6.0			48	2.0	3.0			

* Each culture group number represents 8 to 10 experiments.

† Measured under the microscope in 0.1 mm. units.

‡ Tyrode solution was used as control in place of Ringer solution.

a great increase in the proliferation of fibroblasts, but they also appeared to supply the tissues with the necessary nutrient material for prolonged growth (Fig. 1). When the digestion of fibrin was carried to the optimum degree, the growth of the tissues exceeded even that produced by embryonic juice. The fibroblasts that grew in the fibrin digestion products outlived those cultivated in embryo juice and, in

of pepsin. 5, 10, and 15 gm. of fibrin were digested in 200 cc. of N/20 HCl with 1 gm. each of Fairchild's pepsin. In the first case, all the fibrin was transformed into soluble products which remained in solution on neutralizing and boiling. In the other two cases, some fibrin was left and much protein and meta-protein were precipitated

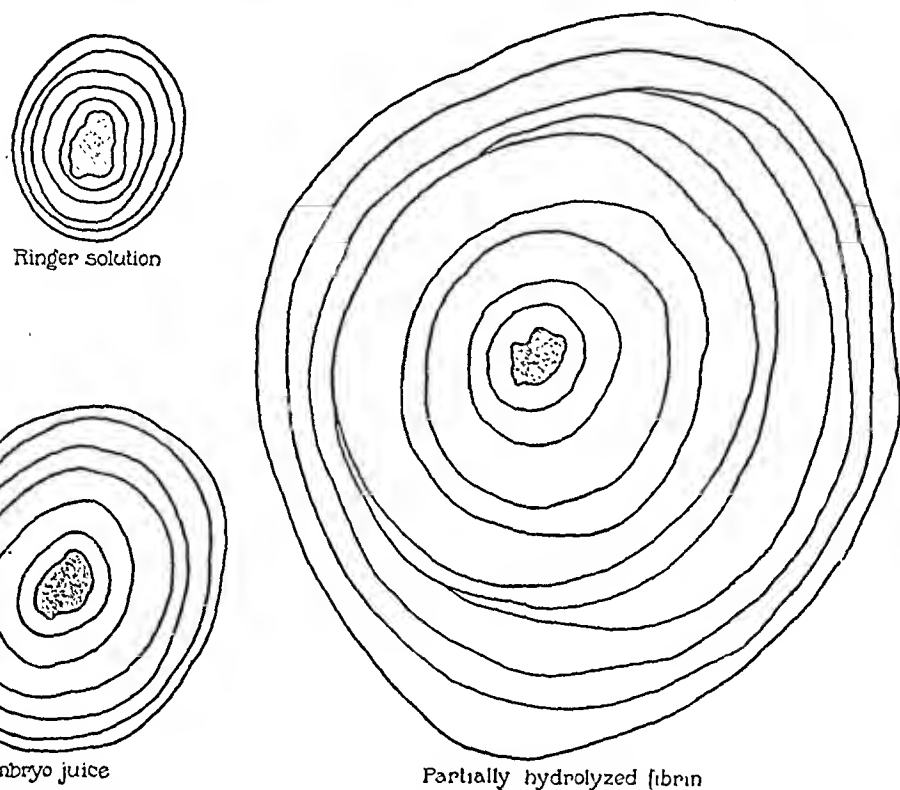


FIG. 3. Experiment 3287 A. Daily tracings under the projectoscope of areas of colonies of fibroblasts cultivated in Ringer solution, embryo juice, and partially digested fibrin. Some contraction of the clot took place on one side of the colony, resulting in a deformation. The growth in partially digested fibrin yielded the largest single colony of fibroblasts ever obtained *in vitro* in this laboratory.

when the solutions were neutralized and boiled. As a basis of comparison, the solutions were diluted to the same concentration of total nitrogen, 0.63 per cent, before being tested for growth-stimulating action on fibroblasts. The amino nitrogen concentration in these solutions was 0.12, 0.10, and 0.07 gm. per 100 cc., giving a ratio of

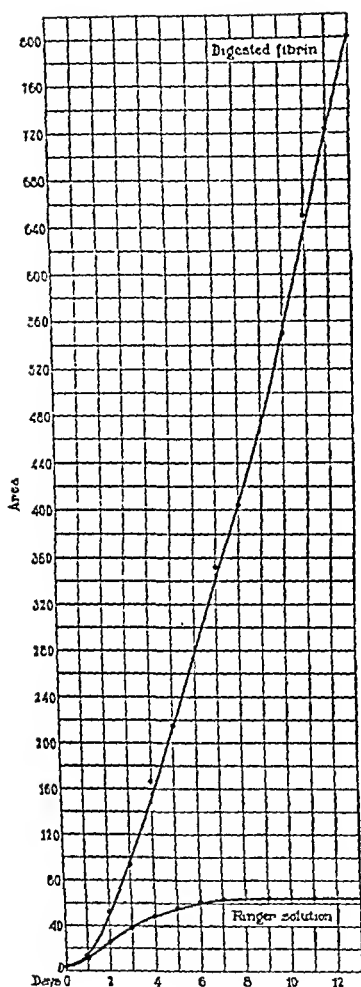


FIG. 1.

FIG. 1. Experiment 3284 A. Growth of fibroblasts in the products of partial peptic digestion of commercial fibrin. The control in Ringer solution died in 7 days. At the end of 2 weeks, the cells cultivated in the digested fibrin were still proliferating rapidly.

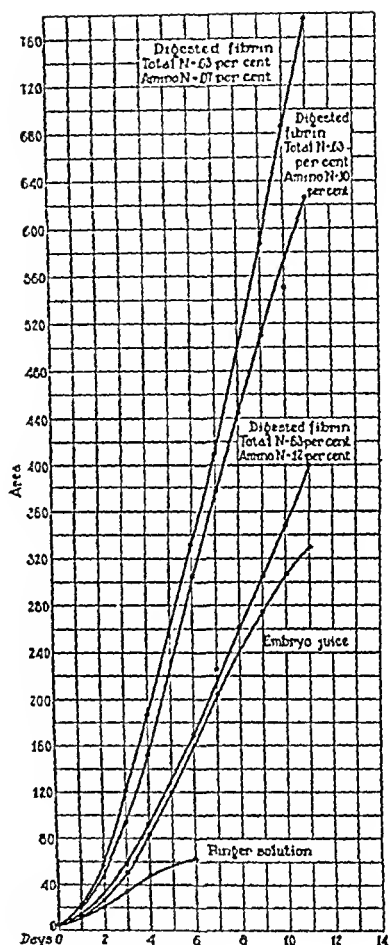


FIG. 2.

FIG. 2. Experiment 3287 A. Comparison of the rate of growth of fibroblasts in three fibrin digests of different degrees of hydrolysis, and in embryo juice and Ringer solution. The three fibrin digests contained the same concentration of total nitrogen, but the ratios of total to amino nitrogen were 9.0, 6.3, and 5.2 respectively. The growth of the cultures in the fibrin digests continued far beyond the points shown in this chart.

complete hydrolysis of these substances produced by autoclaving at 25 pounds pressure for 4 hours yielded toxic or inactive products, thus affording a further indication that the higher cleavage products of protein determine growth.

Action on Fibroblasts of Commercial Peptones.

Commercial "peptones" in solutions from 20 to 1 per cent, sterilized by boiling, were used in the culture medium of fibroblasts.

TABLE IV.

*Rate of Growth of Fibroblasts in Protein Cleavage Products Produced by Autoclaving in Acid.**

Culture group No.†	Chemical preparation No.	Substance autoclaved	Width‡ of new tissue				Time of growth	Ratio: $\frac{\text{Experiment}}{\text{Control}}$			
			Ringer solution	Experiment				Pure	Diluted		
				Pure	Diluted				Pure	Diluted	
					1:2	1:4				1:2	1:4
							hrs.				
3218 A	X-295	Fibrin	1.5	3.0			48	2.0			
3227 A	X-296	"	3.5	5.0			48	1.4			
3235 A	X-296	"	3.0	6.0			48	2.0			
3237 A	X-296	"	1.0	4.0			48	4.0			
3245 A	X-296	"	1.0	4.0			48	4.0			
8689 D	X-296	"	2.0	4.0			48	2.0			
3205 A	X-290	Egg white	2.0	4.0	3.0	3.0	48	2.0	1.5	1.5	
3205 A	X-290	" "	2.0	8.0	8.0	9.0	72	4.0	4.0	4.5	

* 15 pounds pressure for 1.5 hours.

† Each culture group number represents 4 to 8 experiments.

‡ Measured under the microscope in 0.1 mm. units.

Armour's peptone was almost inactive. Parke Davis' and Fairchild's peptones exhibited a small growth-promoting action, while Witte's peptone determined a large growth of fibroblasts (Fig. 5). At a concentration of 5 to 10 per cent, it was toxic. Even in the cultures containing a low concentration of the peptone, the cells showed many fat globules. In spite of this, the rate of growth of the tissues treated with Witte's peptone was as great as that cultivated in embryo juice (Fig. 6). This difference in activity of the commercial peptones is undoubtedly due to the very large concentration of proteoses and the

total to amino nitrogen of 5.2, 6.3, and 9.0, respectively. These solutions were compared with each other and with Ringer solution and embryonic tissue extract, with respect to their growth-promoting power on fibroblasts (Fig. 2). The results clearly indicate that the higher cleavage products are the ones utilized by the tissues. All of these digests produced more rapid and prolonged growth than the embryo juice did. Another pair of digests was compared in which the ratio of total to amino nitrogen was 7.7 and 8.6. In this case, a similar result was obtained. A series having the ratios 8.4, 8.6, and 10.3 showed very little difference, the rate of growth being exceptionally large in all three.

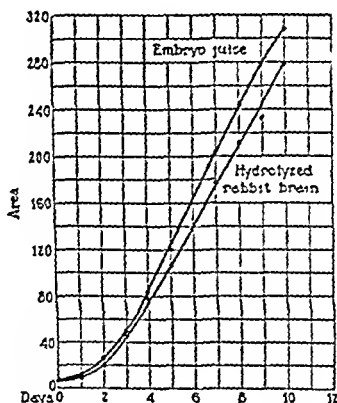


FIG. 4. Experiment 3304 A. Growth of fibroblasts in rabbit brain partially hydrolyzed by pepsin. The growth is comparable to that in embryo juice.

Action on Fibroblasts of Other Proteolytic Products.

Various proteins when partially hydrolyzed appeared to be nutrient for fibroblasts. Two pepsin digests of rabbit brain showed a nutrient action almost equal to that of embryo juice (Fig. 4). These digests gave a strong biuret reaction and contained protein split products having a ratio of total to amino nitrogen of 5.3. The rate of growth produced was comparable to, though less than, that of the fibrin digest having a ratio of 5.2. Hydrolytic products obtained by autoclaving egg white and fibrin in an acid medium at 15 pounds pressure for 1.5 hours proved stimulating to growth (Table IV). More

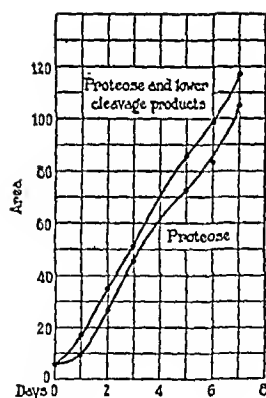


FIG. 7.

FIG. 7. Experiment 3292 A. Comparison of the rate of growth of fibroblasts cultivated in the protease fraction of Witte's peptone and in the fraction containing proteoses, peptones, and lower degradation products. The cells in the protease were in better condition than those in the fraction containing the lower split products, and they multiplied at about the same rate. Concentration of nitrogen equaled 0.398 per cent in both media.

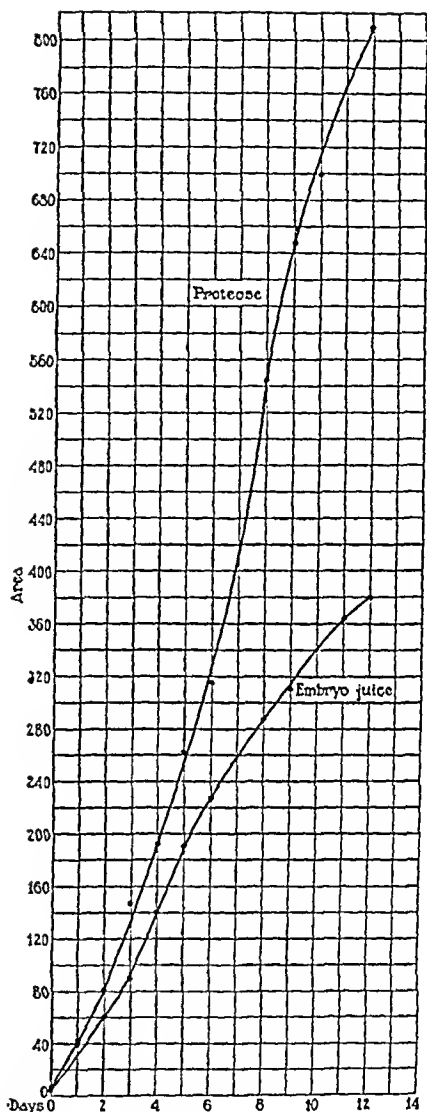


FIG. 8.

FIG. 8. Experiment 8758 D. Growth of fibroblasts in the pure protease fraction of Witte's peptone (0.2 per cent N). The control culture in embryo juice died on the 13th day. The colonies in the protease were still growing after 30 days cultivation.

smaller amount of the lower degradation products present in Witte's peptone in comparison with the others.

Action on Fibroblasts of the Proteose Fractions of Witte's Peptone.

The foregoing experiments indicated that proteoses are probably responsible for the effect of Witte's peptone and other protein digests on the proliferation of fibroblasts. A few preliminary experiments on the fractionation of Witte's peptone confirmed this idea. The meta-

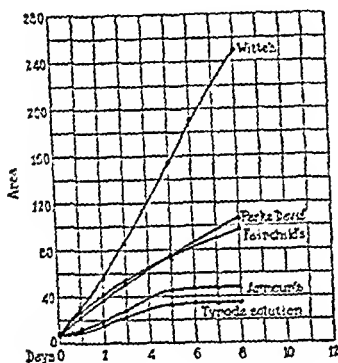


FIG. 5.

FIG. 5. Experiment 3349 A. Action of 2 per cent solutions of commercial "peptones" on the growth of fibroblasts *in vitro*.

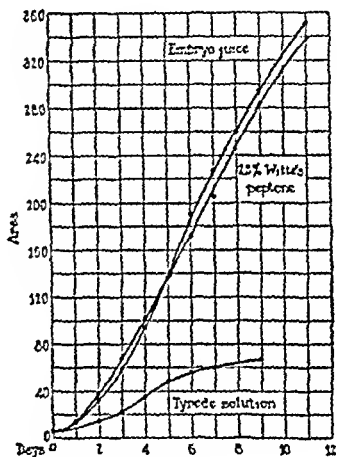


FIG. 6.

FIG. 6. Experiment 3133 A. Growth of fibroblasts in 2.5 per cent Witte's peptone, the control being cultivated in embryo juice.

protein precipitated at pH 6.0 and the substances precipitated by 2.5 per cent trichloroacetic acid had some growth-stimulating action on fibroblasts, but not as much as the remaining fractions. A fraction containing only proteoses was, therefore, prepared by precipitating the higher products, including a part of the proteoses, in 2.5 per cent trichloroacetic acid. The remaining proteoses were separated from the peptones and lower degradation products by saturating at 33°C. with sodium sulfate, after removal of the trichloroacetic acid. This process was repeated four times to insure removal of the other frac-

hydrolyzed by trypsin. The results (Fig. 9) demonstrated that hydrolysis by trypsin greatly reduced the growth-promoting power of the solution, although it did not destroy it completely. The tryptic hydrolysis was not complete, however. It reduced the ratio of total to amino nitrogen from 9.0 to 2.5. Analysis of this tryptic digest showed the absence of proteose except in the smallest traces, and the presence of a considerable amount of peptones and peptides. Therefore, it would seem that the peptones and possibly some of the lower degradation products are nutrient to a certain extent, although they

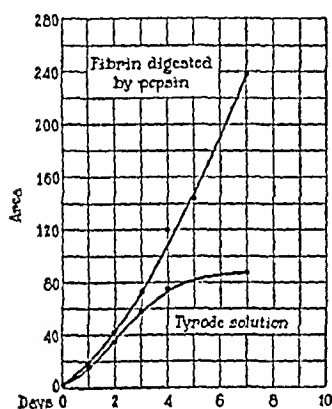


FIG. 10.

FIG. 10. Experiment 3283 A. Growth of a 14 year old strain of fibroblasts in the products of partial peptic digestion of fibrin. Control tissue in Tyrode solution.

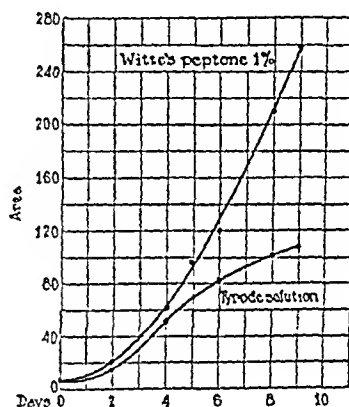


FIG. 11.

FIG. 11. Experiment 8722 D. Growth of a 14 year old strain of fibroblasts in 1 per cent Witte's peptone. Control tissue in Tyrode solution.

do not promote the rapid growth which is produced by the proteoses. All of the amino acids were not present in this tryptic digest in the same concentration as in the peptic digest containing proteoses, for some insoluble products were formed as a result of the hydrolysis. This suggests the hypothesis that the function of the proteose is to furnish a higher concentration of certain amino acids than could be obtained even from their saturated solutions, and to supply them to the tissue cells in a soluble and diffusible form.

tions. The sulfate was precipitated by barium chloride and the solution was finally dialyzed. The effects on fibroblasts of this preparation at 0.398 per cent nitrogen, and of the trichloroacetic acid filtrate containing the proteoses, peptones, and smaller split products, at the same nitrogen concentration, were compared. The rates of growth in the two media did not differ appreciably (Fig. 7), but the cells cultivated in the purified proteose solution showed fewer fat globules in their cytoplasm than those kept in the solution where peptones were still present. In another experiment, a comparison was made of the tissues growing in the purified proteose solution at 0.2 per cent N and in embryonic juice (Fig. 8). The colonies kept in the purified

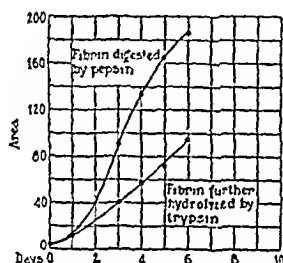


FIG. 9. Experiment 3326 A. Growth of fibroblasts in fibrin partially digested by pepsin and in the same substance further digested by trypsin at equal nitrogen concentration.

proteose grew twice as rapidly as those cultivated in embryonic tissue extract, and the cells contained a smaller number of fat globules.

Evidently, the tissues utilize proteose for building new protoplasm. The concentration of nitrogen in the proteose solutions and in the various protein digests is larger than that of embryonic tissue extract. This undoubtedly means that a part of these nitrogenous substances is not utilized by the tissues. Further fractionation of the proteose is being carried out in order to ascertain whether a more active moiety can be obtained.

Action on Fibroblasts of the Tryptic Digest of the Protein Cleavage Products.

A comparison was made of the rates of growth of fibroblasts in the most active peptic digest of fibrin and in the same solution further

proteoses do, although they appear to be utilized to a slight extent as nutrient materials. The rôle of amino acids, peptones, and proteoses in cell nutrition has not been studied extensively. Burrows¹⁰ found that certain amino acids, even at a low dilution, were toxic for tissues growing *in vitro*. Philippon and Mendeleef¹¹ hydrolyzed serum by sulfuric acid and observed that the tissues displayed more activity in hydrolyzed than in normal serum. Unfortunately, the technique of those experimenters was imperfect, and their results did not indicate whether the medium was nutrient or merely stimulating, and whether the effect could be attributed to a loss of the natural inhibiting power of the serum or to the production in this serum of nutrient or stimulat-

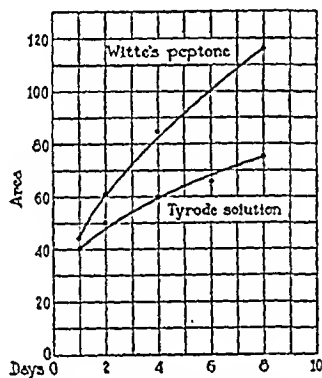


FIG. 14. Experiment 3526 C. Growth of thyroid cells in Witte's peptone. Control tissue in Tyrode solution.

ing substances. Carrel and Ebeling⁴ studied in a quantitative manner the action of artificial mixtures of amino acids on fibroblasts in pure culture, and found that these acids were not toxic and brought about some acceleration of the cell migration without increasing, however, the mass of the tissues. The ultrafiltrate of embryonic juice yielded a similar result, when added to the medium of fibroblasts. These facts have been confirmed lately by Wright,¹² who observed the presence in

¹⁰ Burrows, M. T., and Neymann, C. A., *J. Exp. Med.*, 1917, xxv, 93.

¹¹ Philippon, M., and Mendeleef, P., *Soc. roy. Sc. méd. et nat. Bruxelles, Vol. Jubilaire, 1822-1922*, 713.

¹² Wright, G. P., *J. Exp. Med.*, 1926, xliii, 591.

Action of the Protein Split Products on Pure Strains of Fibroblasts, Leucocytes, Epithelium, and Thyroid Cells.

The foregoing experiments were generally made on fibroblasts that had migrated from fragments of embryonic heart. Similar experiments were repeated on a 14 year old strain of fibroblasts and on pure cultures of pavement epithelium and thyroid cells. Fibroblasts obtained from the old strain were cultivated in various digestion products of fibrin, in Witte's peptone, and in fractions of Witte's peptone (Figs. 10 and 11). The results were similar to those reported above.

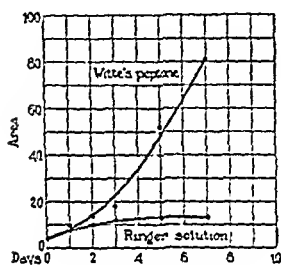


FIG. 12.

FIG. 12. Experiment 3337 C. Growth of iris epithelium in Witte's peptone. Control tissue in Ringer solution.

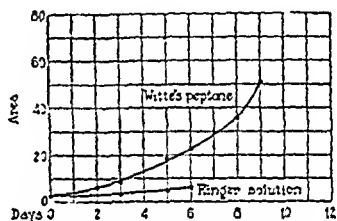


FIG. 13.

FIG. 13. Experiment 3216 A. Growth of guinea pig skin in Witte's peptone. Control tissue in Ringer solution.

Epithelial and thyroid cells as well as fibroblasts were found to multiply with great speed in the presence of the protein split products (Figs. 12 to 14). Leucocytes died when kept in Witte's peptone at a concentration of 2.5 per cent. They utilized it, however, for their nutrition when its concentration in the medium was much lower.

DISCUSSION AND CONCLUSIONS.

These results indicate the nature of the nitrogenous substances required by fibroblasts and epithelial cells for the building up of protoplasm. Tissue cells obtain their nitrogen from proteoses and possibly from some of the other split products of the proteins. The smaller split products do not induce the rapid proliferation of the cells that the

takes place and an increasing amount at pH 5.0. It must be remembered that the fluid contained in the flasks is at a pH of 7.6 and so buffered that it does not change to any great extent during the period of incubation. Under these conditions, the embryo juice enzymes do not manifest their activity, and no proteoses are produced. The pH of the coagulum immediately adjacent to the tissue, however, is modified by the mere presence of living cells. Long ago, Rous observed that tissues growing in a medium containing litmus became surrounded by a pink area.¹³ When phenol red was added to the coagulum in the flasks, the medium as a whole remained alkaline, but a considerable acid production took place around the colonies as shown by the brilliant yellow crown surrounding them. A change to a pH of at least 6.0 occurred at the surface of the cells, which is probably sufficient to enable the tissue juice enzymes to hydrolyze proteins into the proteoses which are absorbed and utilized by the tissues. The objection may be made to this hypothesis that serum is not nutrient for fibroblasts, although it contains enzymes which hydrolyze the proteins in an acid medium. However, it is possible that the removal of the antiproteolytic and growth-inhibiting lipoids would cause serum proteins to be used by the cells, provided a sufficient concentration of enzyme remained. Or it may be that the configuration of the molecules of the protein of embryonic juice is such that they are attacked by cell enzymes which have no effect on plasma proteins.

It has been considered as probable that embryonic juice contains, together with nutrient substances, a specific hormone essential to cell division. The fractionation of the juice did not give any evidence of the existence of such a hormone. Although amino acids or dialysate products had a stimulating effect on cell migration and multiplication,^{4, 12} the substances found to support continuous growth consisted of proteins. The experiments described in the present article render it probable that for cell multiplication a specific hormone is not required and is not contained in embryonic juice, since growth-promoting substances can be obtained from proteins of many sources. While proteoses undoubtedly furnish the nitrogenous materials required for cell multiplication, the fact remains that these products

¹³ Rous, P., *J. Exp. Med.*, 1913, xviii, 183.

embryonic juice of substances which pass through a collodion membrane and stimulate cell division. So far, no increase in the mass of a tissue in pure culture has been determined by a mixture of amino acids, or by the ultrafiltrate of embryonic juice. On the contrary, the larger fragments of the protein molecules can be used as food by tissue cells.

These facts agree with the results previously obtained by the fractionation of embryo juice.³ In those experiments, it was found that fibroblasts do not feed upon the amino acids and other ultrafilterable constituents of the embryo juice, but on the protein fraction. It might be supposed that the protein was utilized as such by the cells, or was

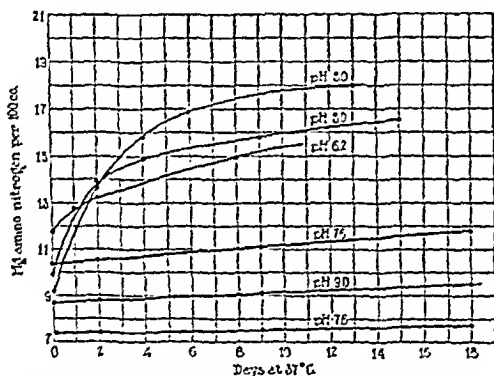


FIG. 15. Increase of amino nitrogen at pH 5.0 and 6.2 in embryo juice incubated at 37°C., showing the presence of a proteolytic enzyme.

hydrolyzed before being absorbed. Many attempts were made to obtain a fraction more active than the original embryo juice. The results of these experiments were negative, probably because the growth-activating substances are not preformed in the embryo juice but are continuously made from its proteins by the cells themselves or by an enzyme contained in the medium. It is a plausible hypothesis that an enzyme contained in the tissue juice becomes active in the presence of living cells. When embryonic tissue juice is incubated at 37°C., at a pH varying from 7.6 to 9.0, no increase in amino nitrogen is observed. But the quantity of amino nitrogen increases rapidly as the medium is made more acid (Fig. 15). At pH 6.2, some hydrolysis

have been obtained from impure proteins and, therefore, other substances may be present which have some action on tissue growth. The study now under way of the digests of pure proteins may show what other products, if any, beside the proteoses are essential to the proliferation of epithelial cells and fibroblasts.¹⁴

SUMMARY.

1. Fibroblasts and epithelial cells in pure culture obtain the nitrogen, which they build into protoplasm, from proteoses and possibly other primary derivatives of proteins. These proteoses have been prepared from embryo tissues, egg white, commercial fibrin, rabbit brain, Witte's peptone, etc.

2. The presence in embryo juice of a hormone that stimulates cell division is improbable.

3. Proteoses separated from peptic digests of fibrin by sodium sulfate determine a more abundant and prolonged multiplication of the fibroblasts than is produced by embryo juice. Peptones and the smaller split products appear to furnish some nutrient material, but do not cause the rapid proliferation characteristic of proteoses, and are sometimes toxic for tissue cells.

4. Possibly the effect of embryo juice on fibroblasts and epithelium is due to the splitting of the protein of the juice into proteoses by the cell enzymes, or by other enzymes activated by the presence of living cells.

The authors wish to express their thanks to Dr. Michael Heidelberger for his criticisms and suggestions in connection with this work.

¹⁴ Since this paper was written, a study has been begun of the products of the partial peptic digestion of crystalline egg albumin. The albumin was recrystallized three times to insure removal of all other substances. A peptic digest of this material, having a ratio of total to amino nitrogen of 4.7, was found to promote the growth of fibroblasts in the same manner as the proteolytic products of the impure proteins. This fact demonstrates that the action of the peptic digests reported above is due to the higher protein split products, and not to any accompanying impurity.

by proliferation of the thymus reticulum cells (4). These cells are endodermal in origin and are formed by a transformation of the epithelial cells of the original thymic ducts to a ramified form. Hassall's corpuscles first appear in the human thymus at about the 3rd month of embryonal life.

Methods.

Young guinea pigs from 30 to 45 days of age were operated upon under ether anesthesia, with strict asepsis. To remove the thymus, a midline incision was made extending from the angle of the lower jaw to the manubrium, the fascia was incised, and both lobes of the gland were removed and immediately placed in sterile physiological salt solution kept at about 38°C. The abdominal wall was then prepared for transplantation by making a midline longitudinal skin incision, reflecting the skin, and with a cataract knife making large pockets between the muscle and fascia. The surrounding fat and areolar tissue were dissected from the thymus and each lobe was introduced into a pocket, the mouth of which was then closed with a silk ligature.

The transplants when removed were immediately fixed in Zenker-formol or Bouin's solutions. The tissues were embedded in paraffin, cut serially, and stained with hematoxylin-eosin. Zenker-formol fixation was not as satisfactory as Bouin's for obtaining good pictures of reticulum cells and mitotic figures.

Histological Findings.

Transplants were studied at 24 hour intervals from the 1st to the 14th day, the subsequent stages being 16, 21, 30, 62, and 120 days. The histological appearance of the transplant for a given day will vary with many factors, such as the size of the lobe transplanted, its vascularization, the thickness of the transplant in the region through which the section was taken, and the age of the animal. Therefore the interpretation of the histological picture necessitates consideration of these factors, and a composite picture of the successive stages of degeneration, regeneration, and growth of the thymus can be drawn only after a study of many serially cut transplants. On the whole, the conditions are as follows:

1 Day.—The thymic lobules are large, and the transplant is degenerating. A peripheral zone of thymus cells 10 to 12 rows deep, still quite normal in histological appearance, remains. The reticulum cells in this zone are actively phagocytic, and are ingesting the neighboring small thymic cells. The most pronounced degenerative changes are seen in Hassall's corpuscles, which show diminished staining and disappearance of the nuclei, and fragmentation of the bodies. The round thymic and reticulum cells in the central part of the transplant show ir-

AUTOPLASTIC THYMUS TRANSPLANTS.

II. WITH PARTICULAR REFERENCE TO THE REGENERATION OF THE RETICULUM CELLS AND THE FORMATION OF HASSALL'S CORPUSCLES.

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PLATES 17 AND 18.

(Received for publication, June 4, 1926.)

In spite of the great number of studies dealing with the thymus gland, no unanimity of opinion has been reached in regard to the formation and fate of Hassall's corpuscles. We are reporting the facts concerning the formation of these corpuscles as we have found them by studying the regeneration of autoplasmic transplants in the guinea pig. This animal's thymus normally contains large numbers of Hassall's corpuscles and is therefore suitable material for such a study.

A review of the thymus transplant literature, for which the reader is referred to a previous paper (1), shows that this gland has been successfully autotransplanted subcutaneously, intramuscularly, subperitoneally, intraperitoneally, and into the spleen. Previous studies of thymus transplants have yielded little information concerning the thymic elements, among them Hassall's corpuscles, because the regeneration and growth of the transplants were not traced in detail.

The purely morphological literature dealing with Hassall's corpuscles is extensive and will not be summarized. Numerous theories concerning their origin have been developed since 1849 when Hassall first described them (2), but he did not discuss their mode of origin. In recent years two conceptions concerning the origin of these bodies have been recognized:

First. Schambacher advanced the theory that Hassall's corpuscles when fully developed represent the remains of the original endodermal thymic cords and tubules (ducts of Remak), now involuted and hyalinized (3).

Second. Hammar elaborated the theory that Hassall's corpuscles are developed

mass, which has been reduced through phagocytosis. Between the regenerating lobules fibroblasts have appeared.

7 Days.—The transplant appears more lymphoid because of active mitosis of the small thymic cells.

8 and 9 Days.—Small irregular regenerated lobules of thymus appear, in which the lymphoid cells are multiplying by mitotic division. The centers of the lobules are occupied by single or conglomerate Hassall's bodies. When the larger ones are traced serially they extend through the lobules for many hundred micra; their cores are degenerated reticulum epithelial cells. The interlobular spaces are organized by fatty connective tissue and numerous blood vessels have appeared.

10 Days.—In places the hypertrophy of the reticulum epithelial cells is so marked, that the picture resembles stratified skin epithelium; the atypical Hassall's corpuscles appear like the pearls of a malignant epithelioma.

11 Days.—The centers of the regenerating thymus lobules now consist of hypertrophic reticulum epithelial cells with numerous single and compound atypical Hassall's corpuscles, while the lymphoid appearing peripheries show numerous dividing small round cells.

12 to 14 Days.—The thymus lobules, although irregular and smaller than normal lobules, have taken on their final form, but central degenerated epithelial cores are still present.

16 Days.—The reticulum epithelial cells are diminished in number; the central degenerated masses are reduced in size and are passing from the lobules into the interlobular spaces. The first differentiation of the regenerated thymus into cortex and medulla appears.

21 Days.—Regeneration of the thymus is now complete. The lobules are lymphoid in appearance, with numerous dividing small thymic cells. True Hassall's corpuscles appear now, developed from the reticulum epithelial cells in the following way: A spent reticulum epithelial cell swells and the surrounding reticulum cells react by arranging themselves around it. These concentrically arranged reticulum cells hypertrophy in the effort to phagocytose the original injured cell. The process may continue until two or more layers of reticulum cells have been added. In this way the Hassall's corpuscle grows, and as it grows, the center degenerates. The greater the central degeneration, the more active is the response of the peripheral reticulum cells. However, in a 21 day transplant only small Hassall's corpuscles are seen, generally not more than 50 to 75 micra in diameter, and they consist as a rule of a central degenerated cell with a surrounding coat of one or two layers of proliferating epithelial cells.

30 Days.—The lobules are distinctly divided into cortical and medullary zones. The Hassall's corpuscles are larger, and some solid masses of reticulum epithelium are also seen.

62 Days.—The transplant is somewhat involuted and fat appears between the lobules. Hassall's bodies are either small, or large and multiple with central cores of debris in which polymorphonuclears and lymphocytes are embedded. These

regular clumping with pycnosis and caryorrhexis of the nuclei. The vessels of the pocket show hypertrophy of the endothelial cells and are engorged; some veins contain an excess of leucocytes and mononuclear phagocytes; the lymph vessels although engorged contain few lymphocytes. Between the pocket and the transplant there is edema, fibroblastic proliferation, and fibrin with leucocytes and mononuclears embedded in its meshes. Numerous capillaries have developed about the transplant and are penetrating its peripheral zone and vascularizing it. New vessels are also extending in between the lobules of the thymus. No mitotic figures are seen.

2 Days.—Many more new blood vessels have formed about the transplant and the peripheral zone is well vascularized. Here hypertrophied reticulum cells are phagocytosing the small thymic cells, after which they join the large central mass of debris, which now shows greater clumping and agglutination than in the previous stage. All the Hassall's corpuscles in the transplanted tissue have undergone complete degeneration.

3 Days.—The histological picture is essentially like that of the 2nd day, but with greater hypertrophy and phagocytic activity of the peripheral reticulum cells and progressive vascularization of the transplant.

4 Days.—Active regeneration has begun in the peripheral zone which has become much widened through hyperplasia of the reticulum cells, many of which are dividing mitotically. This zone is in some places entirely devoid of thymic round cells, while in other places clumps of these cells with an occasional division figure are seen. Hassall's corpuscles now appear here as spherical bodies measuring up to 150 micra in diameter and extending through the tissue for several hundred micra. The core of such a body consists of a degenerated mass of pinkish staining protoplasmic and nuclear material, arranged somewhat concentrically in wavy strands. Many reticulum epithelial cells surround this core, and are sometimes massed acentrically because of rapid amitotic division at one point.

These Hassall bodies are formed when clumps of actively hyperplastic reticulum cells are spent and degenerate. Concomitantly the surrounding reticulum cells proliferate and clothe the degenerating cells which eventually become the core of the Hassall's body. If the proliferation is acentric, a giant cell-like structure may be formed about the core. These bodies are true Hassall's corpuscles but atypical in histological appearance because they are produced very rapidly.

5 Days.—The reticulum cells are enlarged, polyhedral, and vacuolated. Numerous newly formed atypical Hassall's corpuscles are being added to the central mass of debris. Through active division *in situ* the small round thymic cells are increasing in number. Infiltration of these cells from the outside may be definitely excluded.

6 Days.—The changes are like those described for the 5th day only that the reticulum cell overgrowth has progressed. Compound atypical Hassall's corpuscles now appear as a result of the development of multiple areas of degeneration in the hyperplastic reticulum; these structures slowly join the central necrotic

hypertrophy of the surrounding reticulum cells. The study of an involuting transplant proves the common origin of the atypical and true Hassall's corpuscles, the former being produced when the degeneration of the reticulum epithelial cells is rapid, the latter when it is slow.

DISCUSSION.

Regeneration of the thymus has been studied by other methods.

Jonson investigated the effect of hunger and inanition on the thymus of young rabbits and found, as have others, that the gland rapidly involutes. During involution the gland appears epithelial, and all Hassall's corpuscles degenerate, disappearing in about 4 weeks. After 2 weeks of good nutrition Hassall's corpuscles reappear, but regeneration progresses slowly (5).

Rudberg studying the rabbit thymus following x-ray injury noted degeneration of the reticulum cells which frequently led to the formation of cyst-like structures (6). The mechanism of the formation of these cysts is probably analogous to that of the atypical conglomerate Hassall's corpuscles which appear in a regenerating transplant.

Regaud and Crémieu x-rayed the thymus of cats and found that during the regeneration Hassall's corpuscles reach their maximum development from 8 to 12 days after treatment when they are gigantic and occupy about one-half of the parenchyma (7). About the time the small thymic cells reappear, these gigantic Hassall's corpuscles rapidly diminish in size. From 25 to 30 days after x-ray treatment, regeneration of the thymus is complete when small typical Hassall's corpuscles appear in the medulla. That there should be such similarity between the changes which these authors observed in the rayed cats' thymus and those which we have observed in regenerating transplants is interesting.

Goldner showed that adrenalin, when injected in large doses over short periods of time or in small doses over long periods, caused involutionary changes in Hassall's corpuscles (8).

Fulci, studying the rabbit thymus following cauterization, described epithelial overgrowth during regeneration (9). He believed that both Hassall's corpuscles and the small thymic cells were derived from reticulum cells which he termed "*Mutterzellen*."

Concentric epithelial Hassall's bodies have been described in four recent reports of carcinoma of the thymus. Foot, who has traced the formation of these corpuscles, believes that they are degeneration products of the carcinomatous reticulum cells (10). The tumor which he described contained in the necrotic foci the outlines of innumerable degenerated Hassall's corpuscles, some of which occupied an entire low power field.

leucocytes have been phagocytosed by the proliferating reticulum epithelial cells during the formation of the corpuscles and have not infiltrated these bodies subsequent to their formation.

120 Days.—The transplant is involuted and large areas of fat have appeared between the small lobules. The reticulum is prominent, and numerous Hassall's corpuscles appear—large atypical bodies similar to those present in a 4 day transplant, small well formed Hassall's bodies, and conglomerate Hassall's corpuscles with large central areas of debris.

Summary of the Findings.

When an entire thymic lobe is autoplastically transplanted into the abdominal wall of a young guinea pig, the immediate changes which take place are characterized by degenerative phenomena which begin within a few hours, and affect at this time all but a small peripheral zone of the transplant. The Hassall's corpuscles rapidly degenerate. Within 24 hours the reticulum cells in this peripheral zone hypertrophy, becoming phagocytic. Vascularization proceeds and many newly formed blood vessels surround and penetrate the transplant. By the 4th day the reticulum cells of the peripheral zone increase through hyperplasia, while the retained round cells in this zone are phagocytosed and greatly reduced in number. Small atypical Hassall's corpuscles now develop in the periphery when the rapidly increasing reticulum cells degenerate. The continued hyperplasia and hypertrophy of the reticulum cells render the transplant epithelioid in appearance and at this stage Hassall's bodies appear like the pearls of a malignant epithelioma. The central debris is removed by the phagocytic reticulum cells which are rapidly growing in from the periphery. The excess of reticulum tissue now undergoes degeneration forming conglomerate atypical Hassall's corpuscles. Simultaneously the remaining round cells in the periphery rapidly increase by mitosis. Regenerated thymic lobules are formed and their epithelioid centers show numerous large atypical Hassall's corpuscles which when traced serially extend through the lobules for many hundred micra. When the central debris is phagocytosed, the large epithelial Hassall's bodies pass from the lobules into the interlobular spaces. Finally, regeneration is complete; the lobules are differentiated into cortical and medullary zones, true Hassall's corpuscles appear as a result of the swelling of one or more reticulum cells with

angles, I find myself agreeing with this view. However, the thymus is physiologically active in the body, as evidenced by its regeneration following suprarenalectomy, delayed involution after gonadectomy, rapid involution during certain infectious diseases, and the presence of an enlarged gland in status thymicolymphaticus, Addison's, and Graves' diseases.

Concerning Hassall's bodies, I believe that the fully developed corpuscles have no function, being merely aggregates of spent reticulum cells with central cores resulting from degeneration of these cells. The proliferation of thymus reticulum cells, which results in the formation of these corpuscles, may be brought about when the phagocytic activity of the reticulum cells is stimulated by toxins, bacteria, or the presence of dead or injured cells. This fact is beautifully illustrated when the regeneration of a transplant of a whole lobe in the guinea pig is compared histologically with that of a small piece of thymus. The hyperplasia of the reticulum cells in the former is much more marked because of the greater necrosis which results in a more vigorous reticulum cell reaction, leading to the formation of more numerous and larger Hassall bodies.

A study of the progressive changes in the transplant suggests that the essential functions of the reticulum cells are proliferation and phagocytosis under the influence of noxious substances. When these reacting reticulum cells degenerate, the surrounding reticulum cells proliferate, and Hassall's corpuscles with degenerated cores are formed.

CONCLUSIONS.

1. Regeneration of a thymus transplant is characterized by hypertrophy and hyperplasia of the reticulum cells, leading to the formation of small and large atypical Hassall bodies during the early stages.

2. Regeneration is usually complete by the 3rd week, when the newly formed lobules show differentiation into cortical and medullary zones, and typical Hassall bodies appear.

3. Typical Hassall's corpuscles are also derived from the reticulum epithelial cells.

4. These corpuscles have no function, being aggregates of spent reticulum cells.

It may be gathered that large Hassall's bodies can be formed in the regenerating thymus after a variety of injuries. The common origin of the atypical and the true Hassall's bodies has been proved in this study and their common derivation from the reticulum epithelial cells has been demonstrated, the atypical corpuscle arising when the degeneration of these cells is very rapid.

For a proper understanding of the reticulum reaction of the thymus one must remember that the epithelial thymus anlage is not equivalent in all cases. In the majority of mammals the anlage is of purely endodermal origin, but in some species, as for example the mole and guinea pig, it is purely ectodermal, while in others, such as the pig, it is of mixed ecto-endodermal origin. These differences in the origin of the anlage are eventually reflected in the histological appearance of the formed gland, particularly of the reticulum, which in the guinea pig consists of large epithelial cells tending to form concentric bodies. Ducts are rarely seen. On the other hand, the reticulum cells of the rat thymus show little tendency to Hassall body formation, while ducts are frequently seen.

Cysts and ducts found in the fully developed thymus are either remains of the original primary tubules and cords or derivatives of the reticulum epithelial cells. The only relationship between these structures and Hassall's corpuscles is that the cells, from which both the ducts and Hassall's corpuscles develop, are derived from the secondary epithelial cords. The view that normal Hassall's corpuscles represent the atrophic and hyalinized remains of thymic epithelial tubules and cords is untenable since actual counts of Hassall's corpuscles show that there may be an absolute increase or decrease in their number under certain conditions; they may disappear completely from the thymus and then reappear (transplant); and calculations show that in involuted thymuses of old animals the number of Hassall's corpuscles may be as much as 7 times that observed at birth. The view that Hassall's corpuscles may be formed throughout life must be accepted.

The Function of the Reticulum Cells and of Hassall's Corpuscles.

The feeling has often been expressed that the thymus has no important secretion. After having approached the problem from various

FIG. 3. 10 days. The reticulum epithelial cells show marked hypertrophy, the picture resembling stratified squamous epithelium in places. Hyaline degeneration with the formation of large atypical Hassall's bodies takes place.

FIG. 4. 12 days. Shows a regenerated thymic lobule with a central degenerated epithelial core—large atypical Hassall's corpuscle.

PLATE 18.

FIG. 5. 16 days. The central degenerated cores passing from the lobules into the interlobular spaces.

FIG. 6. 30 days. A completely regenerated lobule containing typical small Hassall's corpuscles.

FIG. 7. 30 days. High power of Fig. 6 showing two small typical Hassall's corpuscles.

FIG. 8. 120 days. An involuting transplant with typical small, typical conglomerate, and atypical Hassall's corpuscles. The corpuscle seen near the center is similar to those in the peripheral zone of a 4 day transplant.

5. The thymus reticulum cells are actively phagocytic, and react rapidly when noxious influences are exerted on the gland.

I wish to express my appreciation for the assistance which Miss Alma Adler has given me during the course of this work.

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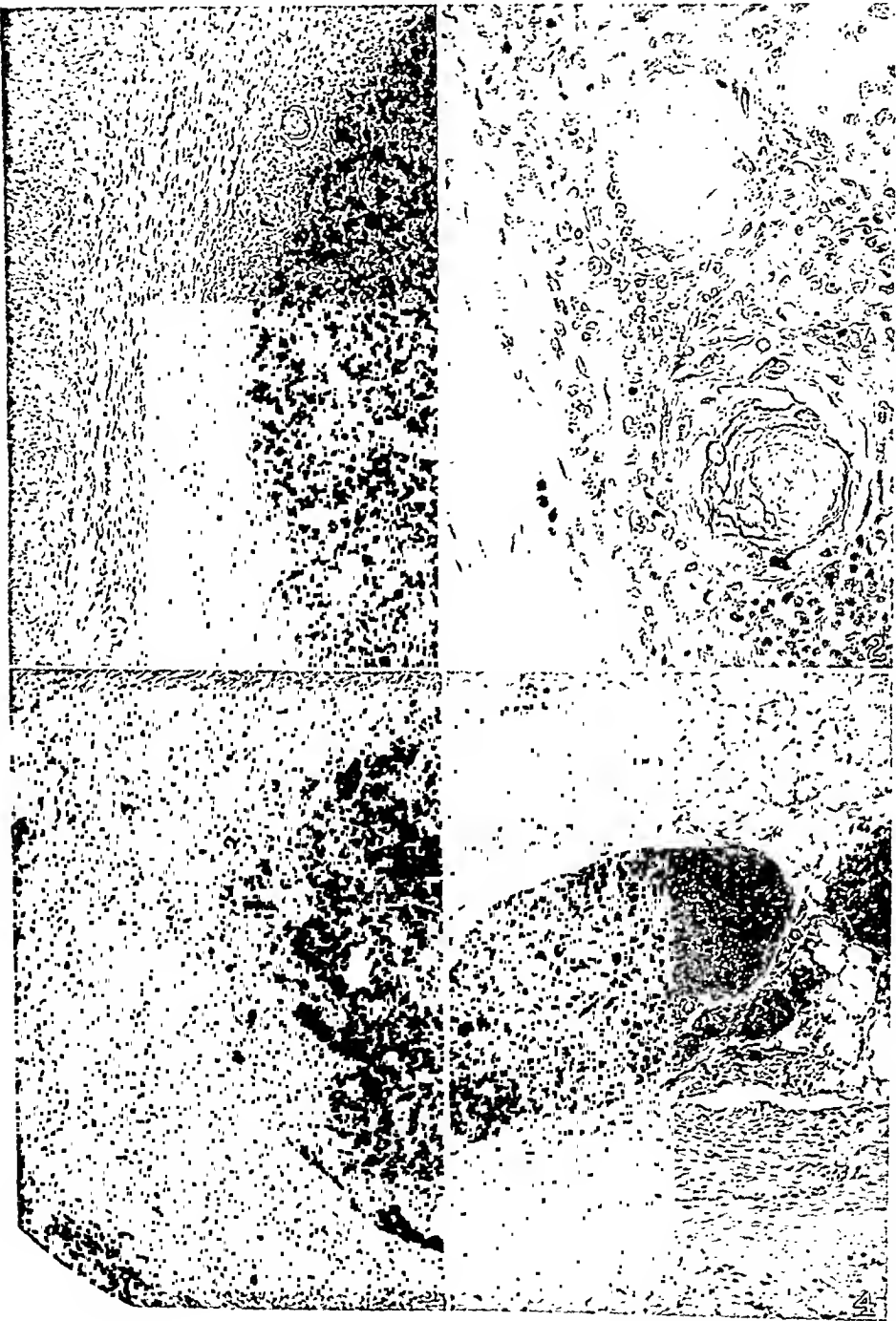
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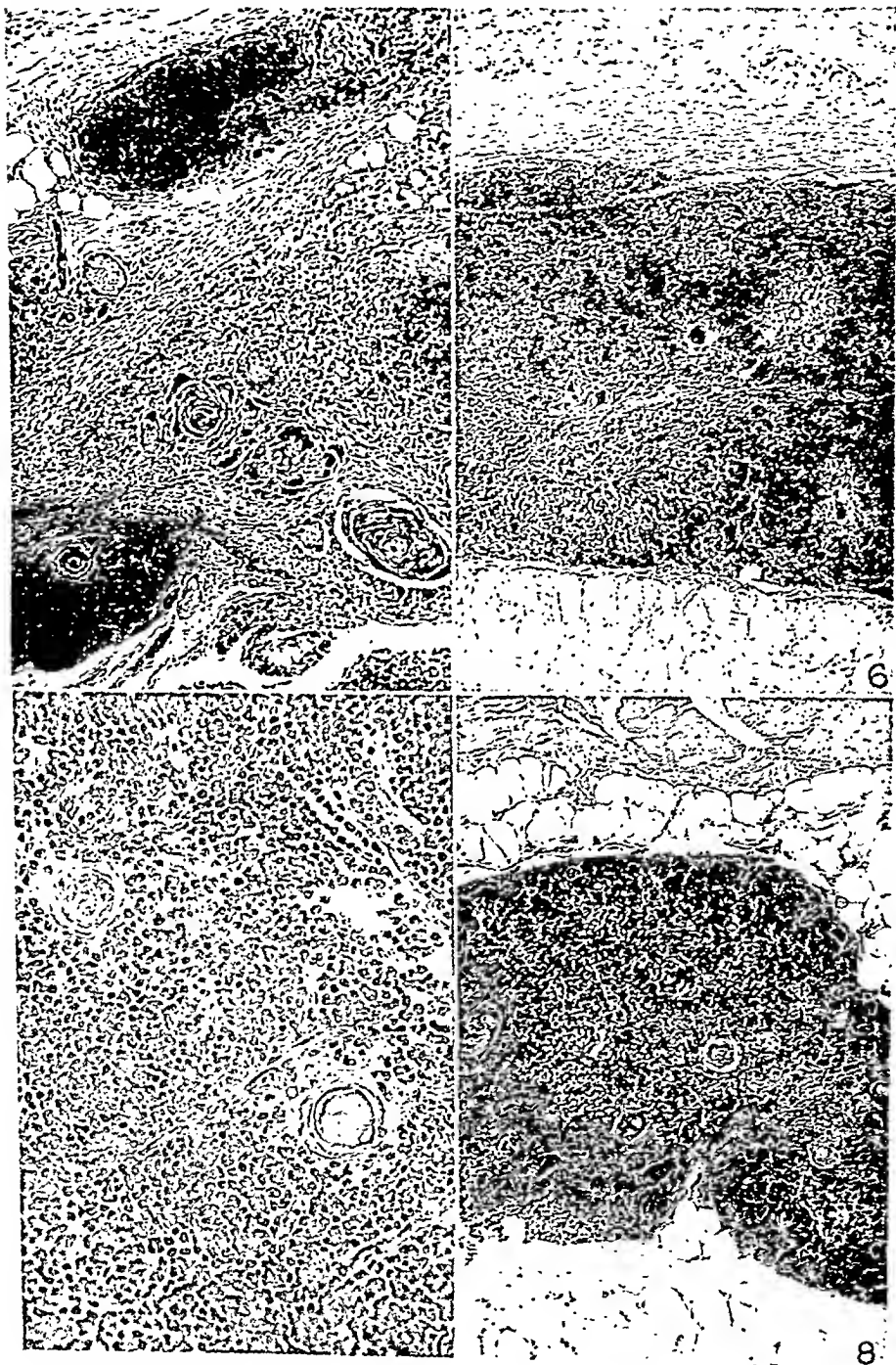
EXPLANATION OF PLATES.

PLATE 17.

FIG. 1. 4 days. Shows, from left to right, abdominal muscle, connective tissue, peripheral zone of transplant with hyperplastic reticulum cells, and newly formed Hassall's corpuscles. To the right of this zone the agglutinated material of the original transplant is seen.

FIG. 2. 4 days. High power of three Hassall's corpuscles in peripheral zone of Fig. 1. One shows a core of degenerated protoplasmic and nuclear material surrounded by reticulum epithelial cells.





(Häufig: Autoplasmic thymus transplants. II.)

rhesus 12,³ were employed (Table II). The temperature of the laboratory where the samples of blood were kept varied from 15° to 22°C., and the tall test-tubes serving as containers were stoppered with cotton plugs and were not protected from the light. There was still a trace of what appeared to be hemoglobin in the supernatant portion of the plasma after 45 days, but the general tone had become brownish

TABLE I.

Viability Tests of Bartonella bacilliformis in Citrated Blood Kept at 4°C.

Source of blood	Date of withdrawal	Result of test on Jan. 29, 1926	Days after withdrawal	Result of test on Apr. 12, 1926	Days after withdrawal
<i>Rhesus</i> 1	Nov. 11, 1925	+	79	+	152
" "	Dec. 8, "	+	32	+	104
" 2	" 18, "	+	42	+	114
" "	Jan. 4, 1926	+	25	+	97
" 3	" " "			+	97
" "	" 25, "			+	86
" "	Feb. 6, "			+	74
" 4	Dec. 18, 1925			+	114
" 5	" 22, "			+	110
" "	Jan. 12, 1926			+	89
" 6	Dec. 28, 1925			+	104
" "	Jan. 4, 1926	+	25		
" 7	" " "	+	24		
" 8	Dec. 18, 1925	+	42		

TABLE II.

Viability of Bartonella bacilliformis in Citrated Blood Kept at Room Temperature.

Source of blood	Date of withdrawal	Titer of fresh blood	Result of test on Mar. 20, 1926 (after 45 days)	Titer of blood on Apr. 12, 1926 (after 67 days)
<i>Rhesus</i> 8	Feb. 3, 1926	1:10	+	> 1:10,000,000
" 12	" " "	1:10	+	> 1:10,000,000

when examination was made 67 days after withdrawal of the blood. As the table shows, the titer of the preserved blood specimens was at least 1:10,000,000, whereas the titer of the fresh blood in each instance had been only 1:10, that is, multiplication had taken place at room temperature. Film preparations made from the specimens showed

³ Noguchi, H., *J. Exp. Med.*, 1926, xlv (in press).

ETIOLOGY OF OROYA FEVER.

II. VIABILITY OF *BARTONELLA BACILLIFORMIS* IN CULTURES AND IN THE PRESERVED BLOOD AND AN EXCISED NODULE OF *MACACUS RHEBUS*.

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Pure cultures of *Bartonella bacilliformis* were first obtained from a specimen of human blood in citrate solution, transported from Lima, Peru, to New York City in the refrigerator.¹ The first successful cultures were made on October 3, and the second on October 20, 1925, the blood being 28 and 43 days old, respectively, when used for the purpose of cultivation. The fact that pure cultures were obtained on both occasions indicated a surprising ability of *Bartonella bacilliformis* to exist under modified conditions and suggested experiments on the viability of the organism.

Viability of Bartonella bacilliformis in Infective Blood.

Table I shows the results of viability tests made with citrated blood of infected monkeys (*M. rhesus*) kept constantly at 4°C. All of the fifteen specimens, which were derived from seven monkeys infected with cultures of *Bartonella bacilliformis* or monkey passage strains, yielded cultures of the organism when tested after periods of refrigeration varying from 24 to 152 days following withdrawal from the animals. In most instances the number of organisms was considerably reduced, as is evident from the fact that none of the preserved specimens yielded growth in dilutions higher than 1:10, whereas the titer in some instances had been 1:100,000 at the time of withdrawal.

In testing the effect of room temperature upon similar material, two specimens of citrated blood, derived from *M. rhesus* 8² and *M.*

¹ Noguchi, H., and Battistini, T. S., *J. Exp. Med.*, 1926, xliii, 851.

² Noguchi, H., *J. Exp. Med.*, 1926, xlv (in press).

an exhaustion of nutrient substances. It was found, however, that the addition, at the beginning of the experiment, of about 10 per cent of fresh rabbit or horse serum and a trace of hemoglobin to the tubes containing the suspension not only prevented the death of the organism but actually induced growth.

Viability of Bartonella bacilliformis in Cultures.

There are two culture media on which *Bartonella bacilliformis* grows well,¹ the so called leptospira medium and blood agar slants. The hydrogen ion concentration most suitable for growth is pH 7.8 to 8, and it is advisable to adjust the medium to this reaction. It has been shown also, through the cooperation of Dr. J. H. Bauer, that the

TABLE IV.

Viability of Bartonella bacilliformis in Culture on Leptospira Medium.

No. of tube	Temperature	No. of days	Result of transplant	No. of tube	Temperature	No. of days	Result of transplant
	°C.				°C.		
1	25	53	+	9	37	37	+
2	25	57	+	10	37	37	+
3	25	63	+	11	37	47	+
4	25	64	—	12	37	47	—
5	25	75	+	13	37	50	+
6	25	80	+	14	37	60	—
7	25	95	+	15	37	62	—
8	25	120	+	16	37	65	—

substitution of Huntoon's hormone broth or Meyer's peptic digest broth for the ordinary meat infusion broth as the basis of these media has a decidedly favorable influence upon the growth of the organism.

One of the peculiarities of *Bartonella bacilliformis* in culture is that it soon reaches the limit of growth at 37°C., though multiplication continues steadily at 25°C. On leptospira medium the organisms remain viable longer than on blood agar slants, growth progressing for a month or more in the former case, while in the latter the maximum seems to be reached in about 14 days at 25°C., and then only when the evaporation of the medium is retarded by soaking the cotton plugs with paraffin or closing the tubes with rubber stoppers. As a rule *Bartonella bacilliformis* remains motile on blood agar for 10 days, occa-

the organisms to be in small clumps scattered among the corpuscles. There was no special tendency to localization within or around the erythrocytes, such as is evident in the blood of human beings suffering from Oroya fever.

Viability of Bartonella bacilliformis in the Nodule from an Animal Experimentally Infected.

Bartonella bacilliformis as it occurs in the local lesions of infected animals is exclusively an intracellular parasite, being found usually in the cytoplasm, occasionally within the nuclei, of proliferating endothelial cells (clasmatocytes). In actively growing nodules the number of microorganisms is very large.

TABLE III.

Viability of Bartonella bacilliformis in the Excised Nodule and Emulsion at 4°C. and at Room Temperature.

Nodule excised on Mar. 1, 1926	Unground tissue		Emulsion	
	4°C.	Room	4°C.	Room
Mar. 7 (7 days).....	+	+	+	+
" 14 (14 ").....	+	+	+	+
" 28 (28 ").....	+	+	+	+
Apr. 4 (35 ").....	+	—	+	—
" 25 (56 ").....	+	—	+	—

The suspension made by triturating with citrate solution a portion of the large subcutaneous nodule that developed on the abdominal wall of *M. rhesus* 14² following the injection of a mixture of culture and passage strain yielded a culture of *Bartonella bacilliformis* in a dilution of 1:100,000. This material was used for determining the viability of the organism in the tissue of the excised nodule. The results, which are recorded in Table III, indicate that *Bartonella bacilliformis* survives in an untritured piece of excised nodule or in the suspension of the tissue for more than 56 days at 4°C. At room temperature, on the other hand, the organisms had died out after a period of 35 days, though growth had been obtained from the suspension after 28 days. The autolysis of tissue taking place at the higher temperature may have been detrimental to the organism, or there may have been

days at the same temperature 0.1 cc. of a 1:10,000,000 dilution of each specimen was sufficient to yield growth. Since the original titer of the blood had been only 1:10, it is evident that *Bartonella bacilliformis* had multiplied considerably under the conditions. Smears of the specimens showed clumps of organisms among the corpuscles but no intracorpuseular multiplication.

Bartonella bacilliformis survived in the excised nodule from a monkey for at least 56 days at 4°C., and for 28 days at room temperature, when a piece of the tissue was covered with citrate-saline solution or ground up in it. The death of *Bartonella bacilliformis* at room temperature under these conditions may be due to the effect of autolysis of the tissue or to a lack of nutrient substances. The suspension alone is not a suitable culture medium, but a trace of hemoglobin and about 10 per cent of fresh horse or rabbit serum make it a favorable one.

The viability of *Bartonella bacilliformis* was tested in cultures kept at 25°, 37°, and 4°C. for varying periods. At 25°C. cultures on leptospira medium remained transferable after 120 days, and when placed in the refrigerator at the time of maximum growth (after 28 days at 25°C.) they were still viable at the end of 4 months. The viability of cultures on blood agar slants depends to a considerable extent upon the care with which the surface of the medium is protected from drying. Under favorable conditions and at 25°C. the organisms remain motile for about 2 weeks and transferable for a month or longer. Cultures on either of these media die out after 50 days at 37°C.

The data presented suggest that it may be fruitful to make a study by cultural methods of pathological material brought from distant parts of the world, even when many days or weeks have elapsed since it was procured.

sionally as long as 14 days, but it is usually non-motile when grown on the semisolid medium. Microscopic examination alone is not sufficient for the detection of viability, therefore; cultural tests must also be made.

Many culture tubes having been preserved from the time of the isolation of *Bartonella bacilliformis* in October, 1925, it was possible to make a series of viability tests, the results of which are recorded in Table IV. In order to retard evaporation, the cultures on leptospira medium intended for preservation were covered with a layer 2 cm. deep of sterile paraffin oil.

As the table shows, a culture of *Bartonella bacilliformis* on leptospira medium at 25°C. remained transferable for a period of at least 120 days, while tubes kept at 37°C. longer than 50 days no longer gave growths on new medium. Culture tubes removed to a refrigerator at 4°C. after their maximum growth had been reached (28 days at 25°C.) were found to be still viable at the end of 4 months.

The viability of the organism on blood agar slants is very inconstant. If the plug closing the tube is not practically impermeable the surface quickly dries, and the organisms cease to multiply and degenerate within a fortnight. On a slant at 25°C. containing sufficient condensation water and closed with a nearly air-tight stopper the organisms remain motile for about 2 weeks and transferable for a month or more. The stopper should not be made absolutely air-tight, because *Bartonella bacilliformis* does not grow in the absence of oxygen.¹ As already stated, a slant culture kept at 37°C. dies out in about 10 days.

SUMMARY.

Fifteen specimens of citrated blood from seven monkeys infected with *Bartonella bacilliformis* were kept at 4°C. for periods of 24 to 152 days, and at the end of each period were tested for viability by the cultural method. All yielded cultures, although there was a considerable reduction in the number of living organisms, as shown by titration.

Two specimens of citrated blood, from infected monkeys, which had been kept for 45 days at room temperature yielded growth when a drop of each was inoculated into leptospira medium; while after 67

This action of the organism on the rabbit accords well with the conception of influenza current at the present time which presents this disease as a mild illness of short duration, characterized by fever, leucopenia, inflammation of the upper respiratory tract, and a marked predisposition to secondary pulmonary infection by organisms of the types usually found in non-influenzal inflammations of the bronchi and lungs. From the standpoint of serious disease and mortality, therefore, the disease may be conveniently regarded as an infectious predisposition to pneumonia. This is apparently exactly the action of *Bact. pneumosintes* upon the rabbit, and taking into consideration the probability of this animal being not as susceptible to the action of this virus as others may be, notably man, the parallelism between their experimentally induced condition, and the natural disease in man, appears sufficiently striking. The probability of the relationship to influenza of *Bact. pneumosintes* is increased by the results of agglutination tests on the blood of convalescents performed by Olitsky and Gates (1921-22) in recent outbreaks which have given apparently specific results.

The general attitude of the profession with regard to this work, however, remains one of skepticism, and general confirmation of their findings is necessary before the organism of Olitsky and Gates can be accepted as the specific cause of influenza. This attitude on the part of the profession is perhaps partly due to the reaction from the too great ease with which the conclusions of Pfeiffer were accepted a generation ago, and the desire to be more than sure that a similar error is not committed at this time. Confirmation of their findings has been attempted by many, a few of whom have published their results. At least partial confirmation has been had at the hands of Loewe and Zeman (1920), who have isolated an organism apparently identical to *Bact. pneumosintes* from influenzal cases. Gordon (1922), Lister (1922), and Detweiler and Hodge (1924) have also partially confirmed this work. Others have failed in similar attempts. Certain details of the work have been criticized by several writers, notably the production of characteristic lesions in the rabbit (Maitland, Cowan, and Detweiler, 1920). Owing to the time-consuming and technically difficult procedures involved in the isolation of this organism, and the fact that only at times of epidemic outbreaks is undoubted influenzal

THE RELATION OF BACTERIUM PNEUMOSINTES TO INFLUENZA: A STUDY WITH A STRAIN OF THE ORGANISM DERIVED FROM THE NASOPHARYNGEAL WASHINGS OF A CASE OF INFLUENZA.*

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The laborious and carefully planned researches of Olitsky and Gates on the etiology of influenza, initiated during the great outbreak of 1918, and continued to the present time (1923) through several epidemic recurrences, have opened up an entirely new field in the investigation of this disease and of others of as yet unknown causation. The details of their findings are too well known to require detailed recapitulation. Suffice it to say that they have isolated from patients in repeated outbreaks of the disease, a minute, anaerobic organism, cultivable with difficulty, which when injected intratracheally into rabbits produces lesions which they believe to be specific and which resemble the pulmonary lesions of influenza as well as these can be deduced from the large amount of autopsy material on record. Animals infected with this organism (intratracheally) have, in their hands, proven susceptible to pulmonary localization of concurrently present microorganisms of the common respiratory types, with the production of typical pneumonias, whereas the same organisms, in animals without *Bacterium pneumosintes*, if producing infection at all, fail to localize in the lungs and cause instead general septicemias. The clinical symptoms produced by *Bact. pneumosintes* alone are very inconspicuous, consisting in a rise in temperature, combined with a leucopenia believed by them to parallel that of influenza¹ and occasionally conjunctivitis, but without other evidence of illness on the part of the animal.

* Published with permission of the Surgeon General of the U. S. Army, who is not responsible for any opinion expressed or conclusions reached herein.

¹ The leucopenia observed in rabbits by Olitsky and Gates after *pneumosintes* inoculation is characterized by a relatively great reduction in the number of lymphocytes. Most reports on influenza describe the leucopenia in that disease as showing a relative reduction of the polynuclear neutrophils and increase in the proportion of lymphocytes.

EXPERIMENTAL WORK.

The nasopharyngeal washings from this case were shaken with sterile glass beads to a uniform emulsion, and the resulting turbid fluid, which had about the opacity of the Army triple vaccine was used for animal inoculations. One rabbit and two guinea pigs were inoculated intratracheally, of which the two latter promptly died.² One guinea pig was inoculated with the unfiltered washings subcutaneously on account of the writer's earlier results with influenzal filtrates injected subcutaneously and intravenously (Hall, 1920). A rabbit was also inoculated with the citrated plasma from the blood of the patient. The results in these experiments will be recapitulated briefly.

First Animal Passage.

The rabbit inoculated with the citrated blood plasma from the patient showed a slight elevation of temperature on the 2nd day after inoculation but no leucocyte reaction. It was not killed until the 5th day. The lungs appeared normal in the gross but on microscopic examination showed a diffuse edematous thickening of the alveolar walls. The results, while perhaps suggestive, seemed so doubtful that no further work was done with material from this animal.

Rabbit 1 received intratracheally 3 cc. of the unfiltered nasopharyngeal washings from the case described. On the 2nd day there was a rise of temperature (1°F.), and a drop of 25 per cent in the total leucocyte count and of 40 per cent in the number of mononuclear leucocytes. The animal was killed and examination of the lungs showed a well marked diffuse lesion of the type discussed later. There was no microscopic evidence of pyogenic infection although cultures from lung and trachea were positive for *B. lepi-septicus*. Portions of the lung emulsion from this rabbit were carried on in two rabbits and one guinea pig.

Guinea Pig 1 received 2 cc. of the unfiltered nasopharyngeal washings subcutaneously. It showed no temperature reaction. On the 4th day there was a definite drop in the total leucocyte count (40 per cent) and a somewhat greater fall in the mononuclears. The animal was sacrificed and microscopic examination of the lung showed the same diffuse lesion observed in Rabbit 1. In addition there were areas of the chronic proliferative lesion described by Maitland and so commonly seen in guinea pigs. The lung of this animal was preserved for several days in 50 per cent glycerol in the ice box and then emulsified and injected into Guinea Pigs 2 and 3. Cultures of fresh and glycerolated lung tissue (chocolate agar) remained sterile.

² Olitsky and Gates gave up the use of guinea pigs on account of the difficulty of successful intratracheal inoculation with this material, and on account of the great frequency of concurrent pulmonary infections in these animals. All intratracheal inoculations were done by operative exposure of the trachea under ether anesthesia, the injection being done with a fine curved needle.

material available for experimentation, it is perhaps not to be wondered at that confirmation, even if ultimately forthcoming, should be slow in appearing. The attempt to repeat the work of Olitsky and Gates in its entirety is manifestly beyond the resources of most laboratories, and it therefore seems justified for the individual investigator to take up special features of their work and such fragmentary observations as are secured in this way should be placed on record to the end that a final decision as to the standing of *Bact. pneumosintes* may be reached as soon as possible. It is the purpose of the present report to record a study of the clinical effects and pathological lesions observed in experimental animals after inoculation with influenzal material, and the results of some attempts to cultivate the organism.

During February, 1922, an epidemic of acute respiratory disease occurred in the city of Washington. It was clinically typical influenza of a type in general greatly milder than that observed in 1918, but was accompanied by a very distinct increase in the incidence and mortality of pneumonia in the city at large. At the neighboring military post of Fort Myer, the epidemic, while unaccompanied by any fatality, ran a characteristically explosive course which, with the equally characteristic clinical characters of the disease, left no doubt in the minds of observers as to the nature of the outbreak. From one of the cases of this epidemic samples of blood and of nasopharyngeal washings were taken February 17, 1922. This soldier was at the time within 24 hours of the onset of the disease. He complained of a sudden onset, severe headache mainly postocular, sore throat, hoarseness, marked pain in bones and muscles, and great prostration, this last persisting for several days after defervescence. His temperature at the time the material was taken was 102.6°F., and his blood contained 5500 white cells per c.mm., of which only 47 per cent were polynuclears. His temperature became normal on the 2nd day following and except for the weakness which persisted, his convalescence was uneventful. Culture of the blood under aerobic conditions was reported as negative, while cultures from throat swabs and of the nasopharyngeal washings showed the presence of the Pfeiffer bacillus and of *Streptococcus viridans*. Inoculation of a mouse intraperitoneally with the nasopharyngeal washings resulted in the isolation of a pneumococcus of Group IV.

leucocyte reduction. These three all received material from Rabbit 1 which had given a satisfactorily positive reaction to primary inoculation. The two guinea pigs inoculated with material from Pig 1 both showed decided reactions; No. 2 developed fever with sharp drop in mononuclear leucocytes, the total count remaining the same. This pig showed a definite pneumonia due to the organism so commonly found in guinea pig and rabbit lesions, *B. lepiroscopicus*. Pig 3 injected subcutaneously gave a very typical leucocyte curve and a slight rise in temperature. Of the seven animals then, four showed to some degree the *pneumosintes* reaction as defined by Olitsky and Gates, one other showed a doubtful reaction which, however, may be considered positive inasmuch as it was material from this animal which was used in the inoculation of Pig 3, which gave a definitely positive reaction, and from which, as will be shown later, there was isolated an organism which has been identified as *Bact. pneumosintes*. Two of the animals gave negative results. Without going into more detail with regard to individual animals, it may be said that further passage of the virus resulted in practically the same way, a certain number of doubtful or apparently negative reactions occurring in each series of inoculations.

In the discussion of the Rockefeller Institute studies much has been said as to the specificity of the pulmonary lesions described by Olitsky and Gates as occurring in rabbits inoculated with influenzal material and not in control animals. Their original description of the pulmonary findings in rabbits infected intratracheally with *pneumosintes* material, which so far as I know has not been since amplified, is as follows:

“Pathological Effects.—The respiratory organs were affected to the exclusion of all others. No pleuritis or exudate in the pleural cavity was evident. The lungs were voluminous as a result of edema and emphysema and had a mottled hemorrhagic appearance. The hemorrhages on the surface, beneath the pleura, were diffuse or discrete, occupying areas a few millimeters in extent or covering a large part of a lobe. In addition, minute petechiæ were seen scattered over the entire surface. On section of the lungs the cut surface revealed a hemorrhagic edema; it dripped a blood-stained, frothy fluid. The hemorrhages again were either diffuse and large, or discrete and small, in the latter instance being numerous.

“On microscopic section carried through various parts of the lungs the lesions were found to consist (a) of hemorrhagic foci, and (b) of edema and emphysema. The hemorrhages varied in size in accordance with the observed macroscopic

Second Animal Passage.

Rabbit 2 was inoculated intratracheally with unfiltered material from Rabbit 1. This material contained *B. lepi-septicus*. On the day following inoculation the animal showed a rise of temperature which continued until the 3rd day (104.4°F.) when it was sacrificed. It showed during this time a progressive fall in total leucocytes and mononuclears. Microscopically the lung showed a fairly well marked diffuse lesion of the same type as did Rabbit 1, with scattered foci of suppurative pneumonia and areas of old proliferative change.

Rabbit 3 received 3 cc. of the same material as the one last described but filtered (Mandler) and aerobically sterile. There was no thermic or leucocytic reaction. There was no diffuse pulmonary lesion and the experiment was regarded as definitely negative.

Guinea Pig 4 received subcutaneously 3 cc. of the unfiltered lung emulsion from Rabbit 1 which contained *B. lepi-septicus*. There was no thermic or leucocytic reaction and examination of the lung showed no diffuse pulmonary lesion.

Guinea Pig 2 was injected intratracheally with 0.5 cc. of lung emulsion from Guinea Pig 1, unfiltered but aerobically sterile. There was a definite progressive rise in temperature, no fall in total leucocytes, but a decided absolute reduction in mononuclears. The animal was sick with rapid respiration, palpable râles in chest, and cough. Sacrificed on the 2nd day after inoculation. The microscopic findings were those of an acute suppurative bronchopneumonia. There was no evidence of the diffuse lesion seen in the earlier animals. However, the prompt production of a pneumonia without leucocytosis by the introduction of material sterile to ordinary cultural test is suggestive of the action of *Bact. pneumosintes* as described by its discoverers.

Guinea Pig 3 received subcutaneously 2 cc. of the same material as the above. This animal showed a slight rise in temperature and a marked fall in leucocytes, especially the mononuclears. It showed, when sacrificed on the 3rd day following inoculation, a well marked diffuse lesion of the type to be described as characterizing *pneumosintes* animals.

Of these eight animals, one may be thrown out as having received blood plasma rather than nasopharyngeal material. Of the remaining seven which received the latter either in the first or second passage, Rabbit 1 showed a typical *pneumosintes* reaction of fever, and fall of leucocytes after 48 hours. Guinea Pig 1, after 4 days incubation (inoculation was subcutaneous) showed a marked leucocytic reaction but no fever. Of the five animals receiving material in the second passage, Rabbit 2 showed fever and leucocyte drop after 3 days; Rabbit 3 receiving filtered material gave an entirely negative result; Guinea Pig 4 showed a slight rise of temperature after 48 hours but no

extent. Cellular exudation into the alveoli was not observed in this series, nor was hemorrhage or exudate observed in the bronchi. In other words, the findings in this series of cases might well correspond to those produced by an agent of a character similar to that of Olitsky and Gates, but acting with much less intensity, provided always that control animals fail to show the same changes.

Control Experiments.

It is necessary before drawing conclusions as to the presence or absence of a distinctive lesion in the animals studied above to establish the fact that such changes in the lungs as have been observed are the result neither of the method used for killing the animal, nor of the intratracheal injection of non-infectious material. The latter possibility is ruled out if, as has been the case in my series, characteristic pulmonary lesions may be produced by other than intratracheal routes of injection. I have, moreover, in connection with other work, given many intratracheal injections with sterile lung emulsions, filtered and unfiltered, without observing the lesion described above. For these reasons I will confine my attention at this time to a study of the effects upon the conditions observed in the lungs after various methods of killing experimental animals. It would appear too far fetched to assume that a hemorrhagic, edematous, and exudative lesion could occur in normal animals.

Some twenty-five rabbits, and a smaller number of guinea pigs, were studied in the attempt to establish satisfactory control conditions. Animals were killed by the inhalation of chloroform and ether, by subcutaneous injection of potassium cyanide, by pithing, and by the intracardiac injection of a saturated solution of magnesium sulfate. None of these methods produced with uniformity lungs as nearly normal on gross and microscopic examination as the method used by Olitsky and Gates, that of dislocating the cervical spine by a single properly directed blow. This method will occasionally fail to produce immediate death, though paralysis and apparent loss of consciousness result immediately. When this occurs agonal lesions in the lungs are observed. Careful microscopic study of a considerable series of normal animals killed in this way leads to the conclusion that there are definite differences between the lesions thus produced and those found in the positive cases of the influenzal series. It is true that areas of hemorrhage are often observed in these control cases, but in no case has the lesion presented the diffuse distribution found in the influenzal series. The hemorrhage in the control cases is often of interstitial character, indeed usually is so, but the evidence of interstitial

appearance, some being microscopic in nature. The edema was more extensive than the hemorrhages and involved alveoli and interalveolar strands of tissue. The alveoli contained coagulated serum or red corpuscles, mononuclear cells, and also at times polymorphonuclear cells of eosinophilic type and desquamated epithelial cells. The interalveolar strands were infiltrated with mononuclear cells and large cells the foreign nature of which was not always clear. Fibrin was sometimes present in small amounts. The bronchi, also, were at times filled with erythrocytes, exfoliated and degenerated epithelia, and leucocytes. The capillaries were distended with blood."

The authors do not mention any variations in the pathologic picture encountered in their experimental animals, such as would be expected to occur in any series of inoculations with a given virus, nor have they indicated that the lesions produced by *Bact. pneumosintes* isolated in later outbreaks of influenza produced less marked changes than those described. Certain it is that in degree, at any rate, the changes shown in the lungs of the animals that I have indicated as probably positive in the series under discussion do not correspond to the above description. The appearance of the lung on removal seldom corresponded to the description given. Only exceptionally was the voluminous appearance observed. Red or hemorrhagic spots on the surface occurred in all the animals of the series, but these spots varied so greatly in color, shape, and size that no description can be given that can be regarded as characteristic. Moreover, such spots are commonly found in control animals. The moist bloody condition of the section occurred regularly in my series, varying considerably in degree. The microscopic picture of my positive cases corresponded better with the photomicrographs presented by Olitsky and Gates than with their written description. The outstanding feature impressing one at first glance was in every case a *diffuse* thickening of the interalveolar walls. On examination with the 4 mm. objective, this thickening was seen to be due primarily to edema, separating the epithelial linings of adjacent alveoli. The capillaries in the walls were engorged and in some places interstitial hemorrhages were evident. Evidence of alteration of the blood in hemorrhagic areas is often seen. Infiltration by cells of a lymphoid type and also by larger cells resembling endothelium was constant though varying in degree. In the rabbits, scattered eosinophil polynuclears were usually seen. Intra-alveolar edema occurred in all cases but was usually of very limited

Careful examination of this animal anatomically, histologically, and bacteriologically failed to show any cause of death other than an extreme hemorrhagic and edematous lesion of the type described for the influenza animals. The entire lung of this animal was of a red meaty appearance and the elasticity of the lungs must have been entirely destroyed. The animal had exhibited great difficulty in breathing and apparently died of suffocation. So far as I know this is the only rabbit which has succumbed to the uncomplicated action of a virus that could reasonably be interpreted as influenzal. The occasional occurrence of animals unusually susceptible to the action of this virus is perhaps to be expected from the similar occasional occurrence of fulminant cases in man.

In the light of the foregoing considerations, it appears to me justifiable to assume that the series of animals in question had received an agent which is capable of inducing a pathological condition in rabbits and guinea pigs characterized, after an incubation period of 1 or 2 days, by some elevation of temperature and reduction of the leucocytes, especially the mononuclears, and, when killed at this time, also showing a pulmonary lesion which is distinguishable from those accidentally incurred at time of death from other causes.

Cultivation Experiments.

When the opportunity for study of this case arose there was available no suitable ascitic fluid for the preparation of the Smith-Noguchi medium. Tubes satisfactorily controlled for sterility were first available at the time of the second animal passage and attempts at cultivation were made with material from all of the animals of this group. Of these only Guinea Pig 3 gave positive results. This animal had been inoculated subcutaneously with the unfiltered lung emulsion from Guinea Pig 1 which had proven sterile on aerobic cultivation on chocolate agar.

The turbidity of the inoculated tubes became noticeably greater than that in controls after about 10 days at 37°. Microscopic examination of these tubes showed minute coccobacillary forms, Gram-negative, and corresponding to the descriptions of *Bact. pneumosintes* as given by Olitsky and Gates. Subcultures on the same medium resulted in continuation of the growth and after three transfers it became possible to secure a good growth and evident colony formation on blood agar plates incubated in the Brown modification of the McIntosh and Fildes anaerobic jar. Emulsions of this growth readily

edema is entirely wanting. Furthermore, the cellular infiltration of the alveolar walls is absent. The hemorrhage is evidently very recent in point of time and no evidences of alteration are seen in the red cells. Such alterations are frequently observed in the influenzal series. (The presence of large pigmented cells is common in all rabbits as also are the eosinophilic leucocytes.) Animals killed by chloroform or ether give a microscopic picture more nearly approximating that of the influenza series, while those killed by intracardiac injection of magnesium sulfate show in most cases an intense engorgement of the pulmonary capillaries, with occasional hemorrhage into the alveoli.

The conclusion to which we are led by these observations is that the lung of a rabbit or guinea pig is very easily injured with the production of hemorrhagic lesions. That method of killing which produces most nearly instantaneous death without struggle or muscular spasm on the part of the animal seems to show on examination the most nearly unaltered lung tissue. Methods of killing somewhat less than instantaneous will give a greater or lesser proportion of hemorrhagic lesions on examination, the least extensive of these lesions showing an interstitial distribution, confined to the interalveolar walls. Such lesions are never diffuse and are characterized by the presence in the walls of fresh unaltered blood without edema or other evidence of inflammatory reaction in the form of cellular infiltration.

As a test of the validity of these perhaps slight distinctions in the pathology of these lungs I submitted a series of slides to Major George R. Callender, of the Army Medical Museum. These included two from rabbits with the influenzal lesion, one killed by chloroform, one by magnesium sulfate injection into the heart, and one by a blow. He promptly identified the two influenzal slides as an acute inflammatory process of similar nature in the two cases, the one from the animal killed by a blow as practically normal in spite of several localized purely hemorrhagic lesions, the one killed by magnesium sulfate as a passive congestion, while the chloroform case was the cause of some hesitation, but finally was placed as a congestion and edema without evidence of inflammatory reaction. My conclusion that the lesion seen in the influenzal cases is specific is also fortified by a previous observation (Hall, 1920) in which a rabbit inoculated intravenously with the filtered lung substance of another similarly injected with the filtrate of the sputum from an early case of influenza, died at the end of 40 hours.

it is with the production of a septicemia without pulmonary localization. For this reason experiments were planned for the purpose of testing this point. There follows the description of one such experiment.

Three rabbits were placed under observation for several days, daily records being made of temperature, total leucocyte count, and differential count. On a given day two of these animals (Nos. 4 and 5) were injected intratracheally with 2.5 cc. of the unfiltered lung emulsion of Rabbit 6, of the series inoculated with the pure culture of *Bact. pneumosintes* isolated from Guinea Pig 3. Both these animals showed on the following day a drop in the leucocyte count as compared to previous records, which mainly involved the mononuclear elements, and one of them (No. 5) showed a slight rise in temperature. The latter, together with the third animal (No. 7) which had received no *pneumosintes* material, received intravenously 4 cc. of an 18 hour culture of *Pneumococcus* Type I, whose virulence had previously been shown to be insufficient to kill at that dose. The latter animal promptly developed a sharp rise in temperature with a doubling of the leucocyte count which persisted for several days with ultimate decided improvement. The animal was killed and the lungs and blood cultured for pneumococci, and the lungs examined microscopically. The latter showed no significant variation from normal and pneumococci were not isolated. The *pneumosintes* animal which received the pneumococcus also showed on the following day a sharp rise in temperature, 3°F. above previous records, but the leucocyte count remained practically constant until the 4th day when there was a drop to 3600, with a temperature of 106.4°F., and the animal died shortly thereafter. Pneumococci of Type I were recovered from the lungs and blood of this rabbit, and microscopic examination of the lungs showed, in addition to the usual signs of *pneumosintes* infection, diffuse invasion of the interalveolar walls by polynuclear leucocytes, with here and there the formation of minute abscesses, and a small amount of exudation into the alveoli. The rabbit receiving *pneumosintes* alone made the usual uneventful recovery.

This and several other similar experiments appear to bear out the contention of Olitsky and Gates that *pneumosintes* material predisposes animals to pulmonary invasion by organisms which ordinarily show no such tendency.

No attempts were made to determine the filterability of the strain of *pneumosintes* recovered at this time as it appears that this property of the organism has no direct bearing on the question of its relation to influenza, and the time at our disposal was limited. No further cases presented themselves for study owing to the prompt subsidence of the epidemic. No extensive control work on normal individuals has been attempted.

agglutinated in low dilutions with serum prepared against *Bact. pneumosintes* and kindly sent me by Dr. Gates, while failing to agglutinate with normal rabbit serum. Once adapted to growth on blood agar the strain was readily maintained by weekly transplants. Injected into animals this organism produced the same reactions as already described for the nasopharyngeal washings of the patient and for the emulsions of lung tissue. The relatively long sojourn in artificial media resulted in considerable diminution of the activity of the organism so that the proportion of animals injected which could be considered as giving positive reactions was smaller than was the case with the original material but sufficient, I believe, to show the connection between it and the lesions and clinical reactions. I was never able, however, to recover the organism from the lungs of animals so inoculated.

Lung-Injuring Properties.

One of the most telling points in the recorded work of Olitsky and Gates, tending to indicate that the action of *Bact. pneumosintes* on animals corresponds to that of the influenza virus on man, is the property of the organism to which it owes its name, that of so injuring the lung as to predispose an infected animal to pulmonary inflammations. These workers found that the guinea pig was an unsuitable animal for experimental work with *Bact. pneumosintes* on account of the frequency of resultant secondary infection of the lungs by organisms commonly found in the respiratory tracts of these animals. Such secondary infection was more rare in rabbits. However, concurrent infections by common bacteria of the respiratory tract were reported as readily induced experimentally.

If it can be conclusively shown that animals infected with *Bact. pneumosintes* are rendered seriously ill or killed by doses of various secondarily infecting organisms so small that control animals show little if any ill effect from their administration, an interesting point in the chain of evidence connecting *Bact. pneumosintes* with influenza will be made. The common organisms found in the respiratory diseases of man, the pneumococcus, for instance, when injected intravenously, show no tendency to localize in the lungs of experimental animals. When death is induced by large doses of a virulent strain

SUMMARY.

Nasopharyngeal washings from a case of epidemic influenza have proven capable of initiating a pathological change in rabbits and in guinea pigs characterized after an incubation period of 1 or 2 days, by some elevation of temperature, reduction in the number of circulating leucocytes, especially of the mononuclears, and by a pulmonary lesion during the period of reaction, which is distinguishable from those accidentally incurred at the time of death.

From one such animal, in the second passage of the virus, an anaerobic coccobacillus, corresponding in all respects to *Bact. pneumosintes*, was isolated by the method employed by Olitsky and Gates.

This organism also proved capable of initiating the pathological change in animals found after inoculation with influenzal material.

The observation of Olitsky and Gates that the presence of this organism in the lungs of experimental animals predisposes to pulmonary localization of other bacteria with the production of definite pneumonic lesions has been confirmed.

Bact. pneumosintes infections may be induced by subcutaneous injection of infected material.

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Having previously determined that our strain of *B. botulinus* yields its most active toxin in the filtrates of 4 day cultures (4), we prepared such filtrates, and found them to contain approximately 100,000 guinea pig M.L.D. per cc. When incubated for a week with the addition of 0.7 per cent formalin, this so called anatoxin (5), was found to be devoid of all toxicity. The rabbits immunized with the anatoxin yielded sera of high antitoxic value. When pooled rabbit serum was titrated in mice, 0.1 cc. protected the animals against approximately 6000 fatal doses of toxin. Precipitation tests were made with a constant amount

Protocol I.

Flocculation of Pooled Antitoxic Serum by Toxic Filtrates of B. botulinus.

	Amount of antigen	.02 cc. of pooled antitoxic serum			Amount of antigen	.02 cc. of pooled antitoxic serum	
		Filtrate of 3 day culture M.L.D. = .00001	Filtrate of 16 day culture M.L.D. = .001			Filtrate of 3 day culture M.L.D. = .00001	Filtrate of 16 day culture M.L.D. = .001
1	.8	—	—	15	.001	—	—
2	.6	—	—	16	.0008	—	—
3	.4	—	—	17	.0006	—	—
4	.2	—	—	18	.0004	—	—
5	.1	—	—	19	.0002	—	—
6	.08	—	—	20	.0001	—	—
7	.06	—	—	21	.00008	—	—
8	.04	—	—	22	.00006	—	—
9	.02	—	—	23	.00004	—	—
10	.01	—	—	24	.00002	—	—
11	.008	—	—	25	.00001	—	—
12	.006	—	—	26	Control of antigen	—	—
13	.004	—	—	27	" " serum	—	—
14	.002	—	—				

of the serum and a series of dilutions of antigen (4 day toxin) so that the zone of precipitation, if narrow, might not be missed. Toxins of various ages and activity were tested, and the tests were repeated a number of times. All were negative, as shown in Protocol I.

Suspecting some error in technique, we made parallel tests with diphtheria toxin and antitoxin obtained from the New York City Department of Health. The typical flocculation of these mixtures followed, as shown in Protocol II, but the parallel series with *botulinus* toxin-antitoxin remained clear. We varied the hydrogen ion con-

THE NATURE OF THE TOXIN-ANTITOXIN FLOCCULATION PHENOMENON.

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Botulinus antitoxin is a specific remedial agent of experimentally demonstrable therapeutic value. However, in clinical cases of botulism the absence of any reliable criteria for early diagnosis precludes the timely application of the serum therapy. The clinical diagnosis of *botulinus* poisoning is made only from those central nervous symptoms which by their very presence foretell the probable fatal termination of the disease.

In an earlier series of experiments it was found that ingested *botulinus* toxin may be detected in the blood of experimental animals before the onset of unmistakable symptoms of poisoning.¹ The blood of larger animals to which toxin had been fed was injected into mice. The presence of the toxin could be established only after its concentration in the blood of the donor animals had reached a comparatively high level, so that the amount injected into a mouse would contain at least one minimal lethal dose. But the recent publications of Ramon would seem to indicate that it may be possible to detect lower concentrations of toxin by means of precipitation with a specific antitoxin (1-3).

With the problem of early diagnosis of botulism in view, we set out to study the mechanism of the Ramon test. Attempts were made to precipitate 4 day toxin by homologous antitoxin. Ramon had shown that when a series of varying amounts of diphtheria toxin and antitoxin are combined and incubated at an appropriate temperature, there appears a flocculent precipitate in a zone of dilutions which corresponds roughly with those mixtures that are neutral in guinea pig tests.

¹ Bronfenbrenner, J., and Weiss, H., unpublished results, 1922.

tration of bacterial protein. The precipitate in Ramon's tests might result, we thought, not from actual antitoxin content of the serum, but from the presence in the serum of an antibacterial antibody. In order to test this notion, we prepared an antiserum which, in addition to antitoxin, might contain antibacterial antibody.

Protocol III.

The Influence of Antibacterial Antibody upon the Flocculation of Antitoxin by Toxins.

Amount of antigen (filtrate)	.05 cc. of antiserum to the 4 day toxic filtrate (no antibacterial antibody).		.05 cc. of antiserum to the 24 day toxic filtrate (antibacterial antibody present)	
	Toxic filtrate of 4 day culture 100,000 M.L.D./cc.	Toxic filtrate of 16 day culture 100 M.L.D./cc.	Toxic filtrate of 4 day culture 100,000 M.L.D. per cc.	Toxic filtrate of 24 day culture 100 M.L.D. per cc.
cc.				
1,000	—	—	—	—
.750	—	—	—	—
.500	—	—	—	—
.300	—	—	—	—
.200	—	—	—	—
.150	—	—	—	<+
.100	—	—	—	+
.075	—	—	—	+
.050	—	—	—	+
.030	—	—	<+	++
.020	—	—	+	+
.015	—	—	<+	<+
.010	—	—	<+	<+
Control of antigen	—	—	—	—
" " serum	—	—	—	—

Immunization with Filtrates of Old Cultures.

For this purpose, we immunized a new set of animals with the filtrates of 24 day cultures of *B. botulinus*. Although the filtrates contained only 100 guinea pig M.L.D. of toxin per cc., as determined by preliminary titration, they were assumed to contain the products of bacterial autolysis.

The filtrates were incubated as previously with 0.7 per cent formalin for a week, and then employed for subcutaneous injection in rabbits. When the pooled serum obtained from these animals was tested against the filtrate of the 24 day

centration, the concentration of electrolytes, and the degree of dilution, but no specific precipitation resulted within the limits of changes not affecting the serum controls. One sample of *botulinus* antitoxin received from the Department of Health gave a very weak and irregular precipitation, but only when this serum was combined with the filtrate of a 24 day culture.

Protocol II.

Flocculation of Diphtheria and Botulinus Toxins with Their Respective Antitoxins.*

		Diphtheria toxin		Botulinus toxin	
		.5 cc. of a 4 L+ toxin	.2 cc. of a 11 L+ toxin	.25 cc. of 4 day filtrate	.25 cc. of 16 day filtrate
Amount of homologous antitoxin	.3000	—	—	—	—
	.2000	—	—	—	—
	.1500	—	—	— (N.)	—
	.1000	—	—	—	—
	.0750	—	—	—	—
	.0500	—	—	—	—
	.0300	—	—	—	—
	.0200	—	—	—	—
	.0150	—	—	—	— (N.)
	.0100	<+	—	—	—
	.0075	<+	<+	—	—
	.0050	++ (N.)	++ (N.)	—	—
	.0030	++	++	—	—
	.0020	—	++	—	—
	.0015	—	+	—	—
	.0010	—	<+	—	—
	Serum control	—	—	—	—
	Antigen "	—	—	—	—

(N.) is the calculated neutral point.

* In this, as well as in other experiments, ++ = copious precipitation; +, fair amount of precipitation; <+, slight amount of precipitation; —, no precipitation.

Having previously observed (6, 7) that precipitation occurs when *botulinus* antitoxin (obtained from the New York City Department of Health) is combined with bacterial autolysates or with extracts of foods infected with *B. botulinus*, we suspected that the present failure might be attributed to the purity of our antigen, since a filtrate of a 4 day culture could be presumed to contain relatively a low concen-

Immunization with Filtrates of an Atoxic Variant.

The experiments described above suggest that the precipitation of toxic filtrates of *B. botulinus* may be wholly independent of the antitoxin content of the precipitating serum. In order to determine this point, an antiserum was prepared by the immunization of animals with a homologous atoxic strain of the bacillus.

*Protocol IV.**Precipitation of Antibacterial Serum Type "A"* by Toxin.*

		.05 cc. of Type "A" antibacterial serum		
		Toxic filtrate of 9 day Type "A" culture (100,000 M.L.D. per cc.)	Formalinized filtrate of 16 day Type "A" culture (originally 1000 M.L.D. per cc.)	Formalinized filtrate of 24 day Type "B" culture (control)
Amount of antigen (fil- trate)	1.000	—	—	—
	.750	—	—	—
	.500	—	+	—
	.300	—	+	—
	.200	+	++	—
	.150	+	++	—
	.100	++	++	—
	.075	++	+	—
	.050	++	+	—
	.030	++	+	—
	.020	+	+	—
	.015	+	—	—
	.010	+	—	—
	Control of antigen	—	—	—
	" " serum	—	—	—

* This serum contains no antitoxin.

Not every individual organism in a culture of *B. botulinus* is a toxin producer. Single cell cultures produce toxins of various strengths, and occasionally a culture will yield no toxin at all (9). By means of single cell culture (Barber's method), a Type B strain was isolated which failed to produce toxin, but which by morphology, staining reaction, and by agglutination was identified with the mother and with sister strains. When the non-toxic filtrate of this strain was combined with a known antitoxin, flocculation resulted (Protocol V).

culture or against the anatoxin prepared from it, the typical flocculation zone was brought out without difficulty. However, when it was tested against the filtrates of the 4 day cultures which we had used earlier (Protocol I), the second serum gave a definite flocculus, but it was less in amount and restricted to a narrower zone (Protocol III) (8).

The best results were obtained when the ingredients were measured directly, with a micro pipette, and without dilution. The racks were placed in the water bath at 55°C. for 20 minutes, in the 37° incubator for 18 hours, and then in the ice box for 24 hours. Readings were made at the end of each interval, and finally the flocculus was stained and examined for the presence of bacterial growth.

Immunization with Washed Bacteria.

Preceding experiments having shown that an antiserum containing antitoxic antibodies alone will not cause precipitation of a toxic filtrate, but that an antiserum that contains antibacterial antibody in addition to the antitoxin will cause such precipitation, it seemed worth while to determine whether an antibacterial serum wholly devoid of antitoxin would cause precipitation of a toxic filtrate.

A 24 hour culture of a rapidly growing strain of *B. botulinus* was filtered from a beef heart medium through a paper filter to remove the meat particles. The cloudy filtrate was centrifuged and the sediment of bacteria resuspended in saline, and then centrifuged again. Washing was repeated a sufficient number of times to insure the elimination of toxin by dilution. The final suspension of bacteria in saline was then shaken with glass beads with a few drops of chloroform in a shaking machine for 48 hours and filtered. The final filtrate up to 1.0 cc. of autolyzed bacteria was found non-toxic by intraperitoneal injection in mice. This material was used for the subcutaneous immunization of rabbits.

The serum thus obtained did not contain enough antitoxin in 0.1 cc. to protect a mouse against even a single M.L.D. of a Type A toxin. Yet the serum, when combined with the toxic filtrates of cultures of various ages, gave in each case definite precipitations in a comparatively wide zone. It yielded similar reactions in approximately the same zone with anatoxins made from toxins that had originally contained one-hundredth the number of M.L.D. per cc. (Protocol IV). As there was no antitoxin in the system, the precipitation could not have been influenced by antitoxic antibodies.

Immunization with Washed Atoxic Variant.

The flocculation of Type B toxin with the serum of animals immunized by the 9 day filtrate of the atoxic variant of this strain was weak and confined to a narrow zone. If the phenomenon is, as our experiments suggested, actually an antibacterial precipitation, it should be possible to increase the precipitating power of the serum by immunizing the animals with the washed culture of the variant.

Accordingly, animals were immunized with the washed bacteria of the atoxic variant, and the serum obtained was found to precipitate the homologous Type B toxin, but more abundantly and in a wider zone (Protocol VII). This serum contained no protective antibodies.

*Protocol VII.**Precipitation of Antibacterial Serum (of Type "B" Atoxic Variant) by a Type "B" Toxin.*

Amount of antigen, Type "B" toxin 300 M.L.D. per cc.	.05 cc. of Antibacterial Serum "B"	Amount of antigen, Type "B" toxin 300 M.L.D. per cc.	.05 cc. of Antibacterial Serum "B"
.500	—	.050	+
.300	—	.030	+
.200	<+	.020	—
.150	+	.015	—
.100	+	Control of antigen	—
.075	+	" " serum	—

Is the Antitoxin Precipitated?

In specific precipitations, the bulk of the precipitate has repeatedly been shown to be composed mainly of the antibody-carrying globulin. If the precipitate which occurred in the Ramon test took place at the expense of the antitoxin, the latter must have been appreciably diminished in concentration in the supernatant fluid.

We tested quantitatively the antitoxic content of the supernatant fluid of the "indicating tube," at the center of the zone (1), using as antigen a formalinized filtrate, paralleling this with a tube that contained the same amount of antitoxin, but in which a heterologous flocculation was made to take place, since it was considered possible that some of the antitoxin might be carried down by adsorption on the precipitate formed. A third tube contained antitoxin diluted with broth

Filtrates of 9 day old cultures of this non-toxic strain were used to immunize guinea pigs. Since these filtrates were wholly non-toxic, it was not necessary to formalinize them. After an appropriate succession of subcutaneous injections, the animals were tested and found not to be resistant even to a single lethal dose of Type B toxin, and

Protocol V.

Precipitation of a Type "B" Antitoxin by the Filtrate of an Atoxic Type "B" Variant.

Amount of antigen (atoxic filtrate)	.05 cc. of Type "B" antitoxin	Amount of antigen (atoxic filtrate)	.05 cc. of Type "B" antitoxin
cc.		cc.	
.500	+	.050	+
.300	+	.030	—
.200	+	.020	—
.150	++	.015	—
.100	+	Control of antigen	—
.075	+	" " serum	—

Protocol VI.

Precipitation of Antifiltrate Serum (of Type "B" Atoxic Variant) by the Atoxic Filtrate Itself and by a Type "B" Toxin.

Amount of antigen	.05 cc. of antifiltrate serum Type "B"		Amount of antigen	.05 cc. of antifiltrate serum Type "B"	
	Toxic filtrate of Type "B" 24 day culture 100 M.L.D. per cc.	Atoxic filtrate of Type "B" variant 9 day culture		Toxic filtrate of Type "B" 24 day culture 100 M.L.D. per cc.	Atoxic filtrate of Type "B" variant 9 day culture
.500	—	—	.050	—	—
.300	<+	+	.030	—	—
.200	<+	+	.020	—	—
.150	+	++	.015	—	—
.100	<+	+	Control of antigen	—	—
.075	—	+	" " serum	—	—

the serum of the animals failed to protect mice against the homologous toxin. However, the immune serum agglutinated the non-toxic variant and also the bacteria of the toxic mother strain. It flocculated the filtrate of the atoxic variant and at approximately the same point flocculated an old toxin (Protocol VI).

filtrate from the protein by this technique in order to see what effect such treatment might have on the precipitation by homologous sera.

To the toxic filtrate of an old culture, normal hydrochloric acid was added until a precipitate appeared. This was removed rapidly by centrifugation and re-suspended in normal salt solution. The hydrogen ion concentrations of both the supernatant liquid and of the resuspended precipitate were carefully adjusted to neutral. At this point both solutions were clear. The acid and alkali used diluted somewhat the original toxin, so that a similar quantity of water was

Protocol IX.

Effect of Acid Coagulation on Specific Precipitation of Toxic Filtrates.

			.05 cc. of antitoxic-antibacterial serum		
			Supernatant fluid neutralized after acid coagulation	Acid coagulum redissolved in normal saline and neutralized	Control dilution of the original toxin
Titration by pre- cipitation	Amount of anti- gen	.50	—	+	+
		.30	—	+	+
		.20	—	+	+
		.15	—	—	+
		.10	—	—	+
		.075	—	—	+
		.050	—	—	—
		.030	—	—	—
		.020	—	—	—
		.015	—	—	—
		.010	—	—	—
	Control of antigen " " serum	—	—	—	—
Titration of toxicity in mice	Amounts given intra- perito- neally	.0003	Died in <21 hrs.	Died in <21 hrs.	Died in <21 hrs.
		.0001	" " <21 "	" " <21 "	" " <21 "
		.00003	" " 42 "	" " 40 "	" " <21 "
		.00001	Survived	Survived	Survived

added to a sample of the original toxin to serve as a control. The three mixtures were then titrated in mice and by flocculation (Protocol IX).

The supernatant fluid lost only a small amount of its toxin and this toxin was adsorbed on the precipitate and was demonstrable there. However, the supernatant fluid did not precipitate the antitoxic-antibacterial serum. The acid coagulum, redissolved and readjusted to the neutral point, did produce the specific precipitate with the antitoxic-antibacterial serum.

to the same extent. The quantities of the reagents used were those that in preliminary tests had been determined as optimum.

After the usual incubation a heavy precipitate appeared in Tubes A and C; Tube B remained clear (Protocol VIII). The flocculus was centrifuged thoroughly, and the supernatant fluids were titrated for comparison of their antitoxic value by intraperitoneal injection in mice, each of which received a single M.L.D. of toxin. The results are given in Protocol VIII.

It is evident that there is practically no difference in the protective power of the serum before (B) and after its flocculation with its own

Protocol VIII.

		Tube A	Tube B	Tube C
		3 cc. anatoxin <i>Botulinus</i> A 1 " antitoxin " "	3 cc. broth 1 " antitoxin <i>Botulinus</i> A	1.5 cc. broth 1.0 " horse serum .5 " anti-horse serum 1.0 " antitoxin <i>Botulinus</i> A
		Each tube centrifuged at high speed and supernatant fluid carefully removed, combined with toxin in doses shown, and injected intraperitoneally in mice. Effect on the mice:		
Toxin "A"	Amount of the supernatant fluids			
.00001	.01000	Survived	Survived	Survived
.00001	.00500	"	"	"
.00001	.00250	"	"	"
.00001	.00200	"	"	"
.00001	.00150	"	"	"
.00001	.00100	Died in 20 hrs.	Died in 80 hrs.	Died in 40 hrs.
.00001	.00075	" " <36 "	" " 44 "	" " <16 "
.00001	None	" " <16 "	" " <16 "	" " <16 "

antigen (A), except for such small amount of antitoxin as may be carried down with the precipitate by physical means (see Protocol VIII, Tube C). The antitoxin as such appears to take no part in the precipitation phenomenon of Ramon.

Removal of the Precipitable Substance.

It has been shown (17) that acidification of toxic filtrate is followed by precipitation of the protein without reduction of the toxin content. The effort was made to separate the toxic elements of the

of titration. When the composition of the bacterial filtrate changes, the results of titration by precipitation fail to agree with those of the animal test.

We have not undertaken to repeat with diphtheria toxin the tests made with the *botulinus* toxin, but as our experiments progressed, we noted in the literature that discrepancies, giving indirect support to our contention, had actually occurred in the practical use of the Ramon test (12-14). Moreover, Moloney and Weld (15) investigated the neutrality of the indicating tube and reported that a deviation of 300 per cent in either direction from the neutral point is possible; and they observed incidentally that the toxin-antitoxin precipitation in diphtheria bears a relation to the concentration of agglutinins in the serum, and thus confirm our preliminary results (8).

Zingher (16) found that after he had removed a precipitate induced by the formalinization of toxins containing tricresol, the antitoxins gave absolutely no precipitate with corresponding sera, although they remained highly antigenic, a condition which he explains in the light of our findings (8).

The demonstration that the phenomenon of Ramon is a specific antibacterial precipitation explains many discrepancies which have been reported. It indicates also the inapplicability of the Ramon test to the detection of the toxin of *B. botulinus* in the blood of animals fed with the toxin, since it is the toxin itself that is absorbed from the intestinal tract, the accompanying bacterial protein having been digested by the alimentary enzymes.

SUMMARY AND CONCLUSIONS.

1. Animals immunized with the formalinized filtrates of young toxic cultures of *B. botulinus* produce an antitoxic serum poor in precipitins.

2. Animals immunized with the formalinized filtrates of old and partly autolyzed toxic cultures produce an antitoxic serum containing precipitins.

3. Animals immunized with toxin-free autolyzed bacteria produce a serum free from antitoxin but rich in specific precipitins.

4. Animals immunized with the filtrates of an atoxic variant produce a serum free from antitoxin but rich in precipitins for the homologous toxin.

DISCUSSION.

A method for the titration of diphtheria toxin and antitoxin *in vitro* would constitute so great an improvement over the biologic one, both because of the elimination of many uncertain factors connected with all tests on animals, and from the point of view of economy, that the publications of Ramon immediately attracted wide attention and stimulated investigation of its reliability. Reports of its usefulness because of close agreement with titrations by the Ehrlich biologic technique soon appeared in the literature (10, 2, 3), and the method was adopted as a routine procedure in many laboratories.

Our study of the phenomenon in the case of the toxin of *B. botulinus* has yielded results that show the precipitation to be entirely independent of either the toxin content of the antigen or the antitoxic content of the serum. It is found that atoxic filtrates precipitate antitoxic sera, and purely antibacterial sera are precipitated by active toxins, the width of the zone and the amount of the precipitate depending apparently upon the amount of bacterial protein present in the antigen used to produce the immune serum. The conclusion, therefore, that the precipitation in this instance is not due to union of toxin and antitoxin, but that it is a purely antibacterial precipitation is unescapable.

How then are we to account for the close agreement that so many workers have reported between the *in vitro* and *in vivo* methods of titration in diphtheria? The answer to this question is, we believe, to be found in the fact that the production of diphtheria toxin has been standardized to such an extent that almost all laboratories follow the same technique. The preparation of the medium, its reaction, the age of the cultures before filtration, and other factors are almost identical (11), and even the strain is the well known standard Park and Williams No. 8.

As the result of this uniformity of preparation, the relation between the toxin content and the concentration of bacterial protein in the culture filtrates tends to be constant. Consequently, the concentrations of antitoxin and of antibacterial antibody in the sera produced by immunization of animals with these filtrates bear sufficiently fixed relations to one another to secure comparable results by both methods

5. Animals immunized with the washed bacteria of the atoxic variant produce a serum that contains no antitoxin, but is rich in precipitins for the homologous toxin.

6. Removal of the precipitins by flocculation with a non-toxic antigen does not materially reduce the antitoxic value of a serum.

7. Removal of the proteins of the antigen by acid coagulation removes the specific precipitable substance.

8. All the sera that contain precipitins produce the specific flocculus when combined with homologous toxins, anatoxins, or with the filtrates of the atoxic variant. The flocculation is restricted within the type. The amount of the precipitate and the width of the zone vary approximately with the estimated amount of bacterial protein in the antigen that is used for the immunization of animals.

We conclude, therefore, that the toxin-antitoxin flocculation is a specific bacterial precipitation phenomenon.

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The filtrates were obtained from nutrient broth-grown cultures and from saline suspensions of streptococci cultivated upon blood agar slants. The cultures on solid medium were grown for periods ranging from 48 hours to 1 week at 37°C. before they were emulsified in saline and filtered through N or V Berkefeld filters. The leucocytes and temperatures of the inoculated rabbits were noted daily for reaction, and observations were made of the areas injected intradermally. All animals dying as a result of the injections were autopsied and the tissues were studied both grossly and microscopically. Other animals were sacrificed during the course of the experiments and a careful study was also made of the various tissues.

To determine whether the active principle of *Streptococcus scarlatinæ* (lysate) induces a characteristic intradermal reaction and to compare the cutaneous lesion with that produced by culture filtrate (Dick test), a series of experiments was carried out upon the immune and non-immune human subjects as well as upon the normal and immune rabbit. The filtered streptococcal lysate which was employed was prepared in the belly cavity of the immune rabbit and *in vitro* by treating the saline-washed streptococci with homologous immune serum. The culture filtrate used was obtained from nutrient broth in which the scarlatinal streptococci had been grown at 37°C. for periods ranging from 2 days to 3 weeks.

EXPERIMENTAL.

Experiment 1 (Infectivity of Scarlatinal Streptococci).—Nine full grown healthy rabbits were inoculated with 5 mil quantities of 48 hour cultures of *Streptococcus scarlatinæ* (Dick's "Tyler," "Harrison" and one of our own strains). Each animal received the entire surface growth of two 24 hour cultures from blood agar slants which were washed off and suspended in 5 cc. of normal sterile saline. The three culture strains were injected separately in similar amounts into three groups of these animals, through the subcutaneous, intravenous and intraperitoneal routes respectively.

No animal of the series as a result of this first injection developed symptoms of infection though kept under close observation and studied for a period of 2 weeks. The daily temperatures and blood counts remained normal. There was no local reaction at any site of inoculation. Blood cultures that were made from a number of the animals during the 1st week following the injection were uniformly negative.

Two of the animals of this experiment, after an interval of 2 weeks, were again injected with 5 cc. of the same culture as was used for the first injection. No infection ensued nor was there any apparent toxic effect, the animals remaining perfectly well throughout the period of observation.

EXPERIMENTAL GLOMERULONEPHRITIS INDUCED IN RABBITS WITH THE ENDOTOXIC PRINCIPLE OF STREPTOCOCCUS SCARLATINÆ.*

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PLATES 19 TO 21.

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In the present communication we wish to report the results obtained in an effort to produce toxic effects in rabbits with the culture lysate of certain strains of *Streptococcus scarlatinæ*. The work was undertaken because of our previous failure to induce toxic effects in the rabbit either with cultures of the specific organism or the culture filtrate. Furthermore we were unable to infect the rabbit with large amounts of living cultures of scarlet fever streptococci.

The fact that in human scarlet fever there is so frequently a nephritic complication, presumably toxic in origin, also prompted attempts to induce the nephritis experimentally. We assumed that the streptococcus of scarlet fever *in vitro* would yield a soluble toxin since the results of Dochez¹ lead him to conclude that both the natural immunity in human beings and the experimental immunity are anti-toxic in nature.

Three different isolations of the specific hemolytic streptococcus of scarlet fever were employed in our present study of the nature and effects of the toxic principle. Two cultures, one designated "Harrison," the other "Tyler," were supplied us by Dr. Dick of Chicago, while the third culture was one of our own which had been recovered from the blood of a case of human scarlatina.

For the purpose of determining the presence of toxin in culture, culture filtrate, and culture lysate, separate series of rabbits were injected with materials subcutaneously, intradermally and intravenously with varying quantities of each.

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¹ Dochez, A. R., *J. Am. Med. Assn.*, 1924, lxxxii, 542.

It is noteworthy that no animal of the series gave any evidence of toxemia or other reaction following the injections. The intradermal sites of inoculation were negative aside from the non-specific redness noted about the needle point. Animals of this series which were subsequently inoculated with a second dose of the homologous culture filtrate and with 10 cc. of a heavy emulsion of living streptococci showed no ill effects. These experiments proved that large amounts of streptococcal filtrate from cultures grown for 2 weeks in nutrient broth contain no toxic principle for the rabbit. Furthermore the experiments indicate that the scarlet fever cultures employed are not capable of elaborating a soluble toxin.

Experiment 3 (Immunization).—A third lot of animals was immunized against living culture of scarlet fever streptococci for the purpose of using them later in the preparation of streptococcal lysate. Seven full grown normal rabbits were injected subcutaneously with saline suspensions of 48 hour growths of streptococci from blood agar slants. The animals received two injections weekly for a period of 1 month. The first dose was one-half of the growth of a blood agar slant which amount was doubled for each succeeding dose. The last dose was approximately the growths of four slants.

Three animals of the series died during the course of immunization but apparently not from infection as cultures prepared from the blood were negative. Death was probably due to intoxication. 1 month after the last injection of antigen (living culture) the rabbits were tested for the Pfeiffer phenomenon and when positive were used later for the *in vivo* preparation of streptococcal lysate. The highly immune animals of this experiment completely split up the living homologous culture that was introduced into the peritoneal cavity and allowed to remain there for 2 or 3 hours. The material recovered from the peritoneal cavity of these animals formed the bacteriolysate used in Experiment 4.

Experiment 4 (Streptococcal Lysate Production).—Eight full grown healthy rabbits which had been previously immunized were employed in this test. The living cultures of the homologous organism were introduced by syringe into the peritoneal cavity of the immune animals. As much as 50 mls of culture were introduced which amount was the total 48 hours growth upon eighteen blood agar slants. 2 to 3 hours after the intraperitoneal injection a number of the animals was sacrificed and the peritoneal fluid collected and filtered. Other animals of this series were permitted to live in order to observe whether any ill effects would result to the animal from a longer sojourn of the culture in the peritoneal cavity. Microscopic examination of the peritoneal fluid of the animals sacrificed showed no cocci or microorganisms of any kind, and cultures prepared with 1 mil quantities of the removed peritoneal material remained sterile which proved there had taken place a complete lysis of the introduced microorganisms.

Two other animals of Experiment 1 received a second injection intraperitoneally of 10 cc. of the originally used cultures (growth of four blood agar slants). The interval between the first and second injections was 21 days. Both animals appeared toxic the day following the inoculation; however, complete recovery occurred 24 hours later. Two other animals of the series were inoculated intraperitoneally with a suspension of streptococci which was the growth from twelve blood agar slants (24 hour cultures). Within 2 hours after the injection the rabbits were seriously ill, dying 6 and 8 hours later respectively. Cultures made from the blood and other organs at autopsy were sterile.

It would seem in the light of these failures to infect, that the rabbit is refractory to large numbers of virulent scarlatinal streptococci, at least for the strains and in the dosage employed. Though not susceptible to infection after repeated injections of virulent scarlatinal streptococci, the rabbit often shows marked toxic effects as a result of a second injection of the homologous culture, especially when the interval between the first and second inoculations is at least 10 days. While the cultures employed by us are not pathogenic for rabbits they seem to be split up by the animal that has been previously sensitized, in consequence of which a toxic principle is liberated. Undoubtedly the streptococci of the first injection succumb in the animal body, and through the action of the derived disintegration product, the immunity mechanism of the rabbit produces a specific lysin. In this way can be explained the toxic symptoms occasioned in the animal as a result of the second injection of culture. On the other hand, the absence of any immediate or later toxic effects upon the animal that has been previously injected with living streptococci, may be accounted for through the presence of insufficient endotoxin extant at any time to induce symptoms.

Experiment 2 (Culture Filtrate).—A second lot of animals was inoculated to determine if the culture filtrate of *Streptococcus scarlatinae* contained a toxic principle. For this purpose eighteen full grown normal rabbits were inoculated with a single dose of scarlatinal culture filtrate. Sets of three animals each received separately 10 mil quantities of the filtrate intravenously, subcutaneously and intraperitoneally. Another set of three animals each was injected with 0.5 to 2.0 mls of culture filtrate intradermally. The filtrate was prepared from cultures which had been grown in 250 cc. of hydrocele broth at 37°C. for 14 days. Only filtrates of cultures showing a heavy homogeneous bacterial cloud were employed. Filtrates were also used from cultures grown on blood agar slants for 2 days at 37°C. All filtrates were obtained by means of the Berkefeld N or V filter, and tested before using for sterility.

is approximately the product of 1 billion streptococci. The animal first showed symptoms of toxemia 6 hours following the injection, and became paralyzed in the lower extremities 12 hours later. During the illness the temperature ranged from 104.2–108.2°F. The leucocytic count dropped from 15,000 to 8000. The animal died 60 hours after the injection, showing symptoms of uremia.

At autopsy the gross anatomical findings were as follows: Heart, flabby and distended, myocardium pale in color and friable. Liver enlarged, soft and dark red in color. Kidneys swollen, and cortex studded with punctate hemorrhages. Lungs and other organs negative. It is noteworthy that there was no evidence of intercurrent infection.

Rabbit 2.—(Mar. 1.) Received intravenously 10 mls of filtered lysate (Dick's "Harrison" culture) which corresponded to the product of approximately 1 billion organisms. 24 hours after the inoculation the animal became ill. Temperature 105°F. and the leucocytic count 17,000. On the 2nd day after the onset of symptoms paralysis in the hind legs appeared. The fever persisted and the leucocytic count rose to 48,000 the day before death which was 1 month after the inoculation. Cultures from the blood and internal organs were negative.

The autopsy findings were not remarkable except for the kidneys which were swollen and contained greatly enlarged glomeruli. The liver, spleen and heart presented the usual signs of toxemia.

Rabbit 3.—(Apr. 24.) Normal half grown animal was inoculated intravenously with 10 mls of filtered lysate (approximately the product of 1 billion streptococci—Dick's "Tyler" culture). Signs of profound toxemia appeared in 14 hours after the inoculation, at which time the temperature was 107°F. and the leucocytes 22,000. There was no paralysis or exanthem. The animal died 2 days later and at autopsy showed marked gross changes of a toxic nature in the heart, spleen, liver and kidneys. The latter were cloudy and speckled with petechiæ.

Rabbit 4.—(Feb. 26.) Full grown healthy animal which had been previously immunized against *Streptococcus scarlatinæ* (Dick's "Tyler" strain) was injected intraperitoneally with the saline washings of thirty-six blood agar slants of the homologous organism. The interval between the last immunizing dose and the present intraperitoneal injection was approximately 1 month. The animal appeared sick the next day and 2 days later developed paralysis of the lower extremities. Though there was recovery from the acute illness the animal died subsequently (27 days after the intraperitoneal inoculation). At autopsy there were found the usual toxic changes for the internal organs. The gross changes in the kidneys were striking in that they appeared confined to the glomeruli.

Rabbit 5.—(May 26.) Large Angora animal was injected intravenously at 10.30 a.m. with 10 mls of filtered lysate (Dick's "Harrison" strain). No immediate effects from the injection were noted. At 4.30 p.m. (6 hours later) the animal was found dead in the cage but still warm.

Autopsy showed remarkable gross changes only for the kidneys. Both organs were intensely congested, soft and swollen. On section the cut surface presented

The amount of peritoneal fluid recovered from the sacrificed animals ranged from 15 to 30 cc., and depended to some extent upon the quantity of culture fluid originally introduced. As a rule, approximately two-thirds of the fluid volume introduced was recovered from the belly cavity within 2 hours afterwards. The collected peritoneal material was always cloudy and of a fluid consistence. After filtration, which was immediately carried out, there usually formed in the clear filtrate a veil-like clot.

Three of the immune animals of this series which had received 30 mls of an emulsion of viable streptococci intraperitoneally, and were not sacrificed for collection of lysate, developed after 24 hours, symptoms of toxemia. The animals were sick for several days; however, they eventually recovered, and have since received two or more intraperitoneal injections of 30 mls of viable culture without showing any ill effects.

Another immune rabbit of this series which was allowed to live after the intraperitoneal injection of 30 mls of viable streptococci, suddenly developed severe toxic symptoms 6 hours afterwards and died 2 hours later. Previous to the onset of symptoms the animal appeared perfectly well. Cultures prepared at autopsy were negative. Still another immune animal of this series which was not sacrificed for lysate collection after having received 20 mls of streptococci (bouillon suspension) into the belly cavity, showed the first signs of toxic effect 5 days later. Paralysis of the lower extremities developed the day following and the animal lingered for several days, finally dying. Streptococci were not recovered at autopsy. In connection with the animals of Experiment 4 it is significant that in no instance did infection occur following the introduction of massive doses of streptococci into the peritoneum.

Experiment 5 (Toxic Effects of Streptococcal Lysate).—To determine whether the bacteriolysate contained a toxic principle a number of normal half grown and larger rabbits were injected by various routes with different quantities of the filtered lysate. 1 mil quantities (equivalent to the growth of one agar slant) were given intravenously and subcutaneously, and 0.1 mil intradermally. The normal rabbits receiving 1 mil of filtered lysate developed well defined symptoms and signs of toxemia in 8 to 36 hours after the injection. The lethal dose was effective over a period ranging from 6 hours to several weeks. Many of the injected animals exhibiting toxic effects showed a temperature as high as 108°F., leucocytosis of 30,000 and paralysis. In no animal was there noted an exanthem. There was a cutaneous reaction at the site of inoculation in some of the animals that received the lysate intradermally. The autopsy findings in the rabbits that died invariably revealed a swollen and congested condition of the internal organs particularly the kidneys. The protocols of representative animals of this experiment are given below.

Rabbit 1.—(Feb. 22.) Full grown normal animal was injected with 5 cc. of filtered peritoneal lysate which had been previously prepared in the belly cavity of an immune rabbit from Dick's "Harrison" culture. As calculated this dosage

The results of these experiments with the toxic principle of scarlatinal streptococci would seem to show that the skin reaction in non-immune humans which is obtained by intradermal injections of culture filtrate, is after all due to an endotoxin and not to a soluble toxin. A comparison of the reactions shows that the human skin is even more sensitive to intradermal injections of the streptococcal lysate than it is to the bouillon filtrate of the specific culture. While the filtrates from streptococcal cultures which have been grown for 2 weeks in nutrient broth, give rise to the intradermal reaction in non-immunes, the filtrates from 2 to 3 days old bouillon-cultured streptococci fail in our hands to produce a skin reaction. Since the human skin reaction (Dick test) is induced only with the older culture filtrate and equally as well with the streptococcal lysate it may be assumed that the specific exciting agent in each instance is the product of disintegrated streptococcal cells. It would seem that extremely small quantities of the product derived from dissolved streptococci are capable of causing the cutaneous reaction in the human non-immune. Therefore it is reasonable to assume that the products of autolysed dead streptococci which certainly occur in culture are contained in the culture filtrate, and could explain the intradermal reaction following its introduction.

Pathology.

The significant lesion occurs in the kidney of rabbits reacting to the intravenous injection of filtered streptococcal lysate. Apart from the nephritis induced in these animals, the lesions in other organs are those commonly seen for a variety of bacterial poisons; namely, congestion of the smaller vessels, tissue edema and parenchymatous degenerations. The central nervous system was not examined. The degree and character of the experimental streptococcal nephritis depend upon the dosage of the lysate and the length of time the inoculated animal survives.

It is noteworthy that the rabbits injected intraperitoneally and subcutaneously with the lysate often fail to show lesions in the kidney, and when changes do occur in this organ they are comparatively mild and of no special significance. The most striking and significant

a peculiar rose hue suggesting hemolyzed blood. The congestion was distinctly demarkated for the zone intermediate between the cortex and base of the pyramids. The glomeruli were swollen, red and bleeding. Anatomical diagnosis:—acute hemorrhagic glomerulonephritis (fulminating).

Cutaneous Reaction.

To determine whether the lysate of scarlatinal streptococcus induces a skin reaction, and to compare the lesion with that produced by culture filtrate (Dick test) a series of experiments was carried out upon immune and non-immune volunteers as well as upon the normal and immune rabbit. The filtrates of streptococcal lysate employed in the tests were of two kinds; namely, one which had been prepared in the belly cavity of the immune rabbit and the other made *in vitro* by treating the saline-washed streptococci with specific immune serum. The culture filtrates used in the test were also of two kinds; one was obtained from a 2 weeks old bouillon culture, and the other from a broth culture which had been allowed to grow only for 2 days at 37°C.

Experiment 6 (Human).—Six human volunteers, including three known non-immunes, were intradermally injected into different areas on the inner aspects of the forearms with 0.2 mil quantities of the filtered streptococcal lysates and culture filtrates respectively. Each subject received simultaneously into separate areas of the skin the various culture filtrates and lysates under consideration. The right arm was used for the intradermal injection of the filtered lysates and the left arm for the culture filtrates. Similar amounts (0.2 mil) of normal sterile saline and bouillon were separately injected into the skin as controls.

The three non-immunes developed in 24 to 36 hours typical erythematous reactions about the injection sites where the two lysates were introduced. The inoculation sites on the corresponding arm which received the two kinds of culture filtrate, showed only a reaction for the "older filtrate." This reaction while definitely positive was not as prompt in appearing nor as intense in character as was the reaction in the other arm which received the streptococcal lysates. The controls were negative; likewise no typical reaction was noted for the culture filtrate prepared from 2 day old cultures.

Experiment 7 (Rabbits).—Six rabbits were used, four normals and two immunes, for testing the skin reaction to streptococcal lysate and culture filtrate. In the normal animals of the series no reaction occurred for either the lysate or culture filtrate. We were unable to even induce an intradermal redness with 5 mils of the culture filtrate; however, for the immune animals a marked inflammatory reaction appeared at the site of inoculation following the injection of 1 mil dosage of lysate.

evidence for tufts in which shrinkage and complete disintegration have occurred, Bowman's capsule then containing only necrotic remains of vessels and their endothelium. For some glomeruli the necrotic tuft appears shrunken, in shadowy outline and eccentrically displaced by either collections of serum, blood clot or "crescentic" masses of proliferated capsular epithelium. The latter are the so called "epithelial crescents" which invariably begin in the tubule portion of Bowman's capsule and in consequence cause a blocking of the entrance to the corresponding tubule.

Tubular changes are not an early feature of the acute parenchymatous nephritis. Often no epithelial alterations are observed except where the corresponding glomerular tuft is markedly altered. In these instances the tubules show a swollen and granular condition of the lining epithelium especially of the convoluted portion. At this stage desquamation of the epithelium and its appearance in the form of casts are frequently noted.

In none of the experimental rabbits dying in a week to 10 days as a result of the acute nephritis did there occur lesions outside of the parenchyme. In other words, there was no lymphocytic infiltration of the interstitial tissues or proliferative activity of the connective tissue stroma. However, it should be mentioned in this connection that in certain of our experiments where one of the rabbits previously immunized against the streptococcus of scarlatina died as a result of a subsequent injection of the homologous "lysate," chronic interstitial changes were noted in the kidneys. These chronic changes were essentially reparatory and undoubtedly the sequence of some previous injury to the parenchyme. Whether the primary glomerular injury induced experimentally with the streptococcus may inaugurate secondary chronic changes in the stromal tissues can only be conjectured at this writing. Further experiments in which the chronic lesions occur regularly under specific conditions are necessary before any conclusions can be drawn regarding the specificity of the interstitial change or its relationship to the acute glomerular nephritis experimentally produced with *Streptococcus scarlatinæ*.

lesions in the kidney follow the intravenous administration of the filtered lysate.

The gross appearance of the affected kidneys varied from a barely perceptible cloudy and swollen condition to one in which the organ is much enlarged, capsule tense and the cortical substance studded with punctate hemorrhages. On section the cut surface is mottled dark red in color and the normal markings are obscured. The small hemorrhages in the cortex seem to correspond, for the most part, to the location of the glomeruli. Other tufts appear swollen, dark red and elevated. The capsule is not adherent to the cortical substance, nor is there any gross evidence of interstitial change.

The microscopic study of the kidney sections fixed in formalin and Zenker's fluid and stained with hematoxylin and eosin, reveals an acute glomerulonephritis as the outstanding lesion. Occasionally the corresponding tubule is involved though often only the convoluted portion. The glomerular change ranges from a simple acute hyperemia of the tuft capillaries to a marked congestion and serum extravasation and hemorrhage into the capsular spaces. In these instances the capillary whorls appear to be pushed to one side or partly crowded out of Bowman's capsule.

The sections of kidney from rabbits that survive the immediate effects of the lysate, though not completely recovering, show remarkable changes in the Malpighian bodies in the form of hyaline thrombi of the tuft capillaries. Microscopic fields in which there are six to ten glomeruli, show hyaline masses in the vessels of fully one-third of these. The convoluted part of the corresponding tubule often reveals an advanced stage of retrograde metamorphosis for the lining epithelium. No change is noted for the tubules not connected with thrombosed tufts. The epithelium appears structurally normal for tubules in which the glomerular portions present lesions of a mild character.

Still another form of glomerular change is noted for the kidney of paralyzed rabbits which died 3 weeks or more after the intravenous injection of streptococcal lysate. The Malpighian bodies contain a marked increase in the number of mononucleated cells lining the tuft capillaries. The picture is not that of an endarteritis as the cells seem to be free in the lumen of the vessels. These cells are not in

immunization dose it is insufficient to injure the animal. It seems also apparent that but little antitoxin is formed from this small amount of liberated toxin since a subsequent intraperitoneal injection will prove fatal, indicating a failure of neutralization of the toxin. That there occurs marked lysis of the organism injected intraperitoneally is demonstrated by the absence of these organisms in smear preparations of the fluid and negative cultural results.

The bacteriolysate *per se* and the filtrate contain a toxic principle as is proven by the fatal effect upon the rabbit in from 6 to 12 hours. This toxin while violent in its action requires large doses to produce such a result and is, therefore, not comparable in potency to the soluble toxin of microorganisms such as *B. diphtheriæ*.

Since *Streptococcus scarlatinæ* is not pathogenic for the rabbit, it is necessary to employ large doses of the toxin to produce a fatal result. However, this same bacteriolysate is more toxic for the human species as is demonstrated by intradermal injections. Cutaneous injection of 0.2 cc. into the rabbit produces no effect whereas this amount when similarly administered to non-immune human subjects produces a marked reaction. Careful standardization of the toxic bacteriolysate has not yet been undertaken. In our experiments any dose less than 5 cc. has not caused death.

It is questionable whether the skin reaction to intradermal injection of specific culture filtrate in humans who are non-immune to scarlet fever, is produced by a streptococcal exotoxin. There is no experimental evidence in the rabbit, at least, to show that the scarlet fever streptococcus elaborates what we are pleased to call a soluble toxin. Certainly for this animal an active toxic principle is not demonstrable in the filtrate of cultures grown in nutrient broth for periods of 10 days to 2 weeks. The endotoxin of scarlet fever streptococci does not preclude its accounting for the exanthem in the human case or the skin reaction in the human non-immune. If we accept the view that scarlet fever is a localized streptococcal infection, we must recognize the fact that the organisms are constantly dying and being destroyed by the host. In consequence, the liberated endotoxin may reach the cutaneous tissues *via* the circulation in sufficient concentration to give rise to the exanthem. An intracellular poison of a pathogenic microorganism is not different from that of a soluble toxin in its specific action upon the tissues.

DISCUSSION.

The experiments which we have carried out upon rabbits indicate that the active toxic principle of the *H. Streptococcus scarlatinæ* is bound up in the cytoplasm of the microorganismal cell. The experiments also show that the toxin of certain strains of scarlet fever streptococci, including the Dick "Harrison" and "Tyler" isolations, is not a secretory product of the living organism. In consequence of this the active principle is in no way comparable to a soluble toxin.

Primary large doses of the scarlatinal streptococci or the culture filtrate injected into the rabbit do not produce a toxic reaction. After several injections have been given for immunization purposes a subsequent unduly large inoculation of the organism may produce toxic effects. In other words, a dose which given primarily would have no effect upon the rabbit will, on the other hand, produce toxic symptoms in the animal that has been partially immunized. This would seem attributable to the liberation of an intracellular or endotoxin from the injected organism by the specific bacteriolysin previously produced in the animal through the action of specific antigen. It cannot be attributed to any free toxin injected, as the same dose or a larger amount is inert when given as a first inoculation.

After immunization of the rabbit the intraperitoneal injection of a large amount of scarlatinal streptococci will produce toxic symptoms and death in from 2 to 24 hours whereas the intraperitoneal injection of a similar quantity into the rabbit which has not been immunized, has no appreciable effect. Manifestly the endotoxin has here again been liberated by the specific bacteriolysin present in the immunized rabbit and the action of this liberated toxin may prove fatal. In procuring our bacteriolysate from the peritoneal cavity of the immune animal which had been given a large dose of the streptococcal culture into this cavity, it was considered preferable to obtain this toxic material in from 1 to 2 hours following the intraperitoneal injection, in consequence, the animal was sacrificed at the end of this period. Through this procedure the greatest amount of the introduced culture material could be recovered and at a time when its endotoxic content demonstrated considerable potency.

While a certain amount of toxin must be liberated following each

ployed. The degree of the toxic effect upon the rabbit depends upon the size of the dose and the route through which it is introduced. The specific effects range from mild to severe and fatal forms of toxemia as indicated by high fever, leucocytosis, paralysis and acute hemorrhagic glomerular nephritis.

5. The experimentally induced nephritic lesions are analogous in kind and variety to those of acute scarlatinal nephritis in man, including the "epithelial crescent" formation, hyaline thrombi of glomerular capillaries, hemorrhage into capsular space and necrosis of capillary tufts.

EXPLANATION OF PLATES.

PLATE 19.

FIG. 1. Section of rabbit kidney showing swollen and distorted glomerulus. Note the hemorrhage in Bowman's capsule which has displaced the capillary tuft and extends into the corresponding tubule.

FIG. 2. Section of rabbit kidney showing early vascular changes in the glomerular vessels. Note the marked dilatation of the tuft capillaries which are distended with red blood cells that have lost their hemoglobin and appear as sharply outlined colorless circles crowding the vessel lumina. The adjacent convoluted tubules are swollen and the lining epithelium granular.

FIG. 3. Section of rabbit kidney showing atrophied tuft and Bowman's capsule more than half filled with proliferated epithelium ("crescent").

PLATE 20.

FIGS. 4 and 5. Section of rabbit kidney showing various proliferative and degenerative lesions in the glomeruli. Note the marked increase in the endothelium of the tufts and the distention and vacuolization of the latter.

PLATE 21.

FIG. 6. Section of rabbit kidney in which the glomerulus is degenerated and pushed toward the vascular end of Bowman's capsule by extensive hemorrhage.

FIG. 7. Shows hyaline thrombi in glomerular vessels. The thrombi are apparently situated in the efferent loops of the capillary tuft.

FIG. 8. Section of rabbit kidney showing exudative and proliferative changes in the glomerulus. The capillary tuft is atrophied and Bowman's capsule is partly filled with extravasated serum. Note especially that in one part of the capsule there is the early formation of the "epithelial crescent."

Since in human scarlet fever there is commonly an acute nephritis, the production of kidney lesions in the experimental animal with scarlet fever streptococci is of significance. The experimental nephritis herein reported for rabbits supports the view that the streptococcus is responsible for the acute toxic nephritis in scarlatina but not necessarily is it inferred that the streptococcus plays a solitary rôle in the production of the scarlet fever symptom complex.

A study of the histopathology of the rabbits dying as a result of the intravenous injection of streptococcal lysate shows pronounced lesions in the glomeruli of the kidneys. The constant occurrence of the lesion in the kidney tuft suggests a selective action of the streptococcal endotoxin and permits of the deduction that the mortality in the experimental animal is the result of an acute hemorrhagic glomerulonephritis. Furthermore, the location and character of the nephritic lesion of the rabbit indicate that the acute nephritis in human scarlet fever is caused by certain hemolytic streptococci.

Since the character of the kidney lesion in the experimental rabbit is in many respects like that of the acute scarlatinal nephritis in man, it constitutes evidence of the possible specific relationship of the streptococcus; however, it is no proof that the hemolytic streptococcus is the only cause of the disease.

SUMMARY.

1. Broth-grown cultures, cultures from blood agar slants and culture filtrates (Berkefeld N or V) of *H. Streptococcus scarlatinæ* are without appreciable effect upon the rabbit, no matter how large the dose or by what route introduced.

2. The active toxic principle of *H. Streptococcus scarlatinæ* for rabbits is intimately associated with the protein of the bacterial cell, and is not given off in the artificial medium during the growth activity of the organism, indicating, therefore, its endotoxic character.

3. The endotoxin is readily obtained from the viable scarlatinal cultures through the medium of the peritoneal cavity of the rabbit immunized against the homologous strain (Pfeiffer phenomenon). The toxic substance thus obtained we have termed a lysate.

4. The rabbit is highly susceptible to the *in vivo* prepared lysate of *Streptococcus scarlatinæ*, at least from the cultures we have em-

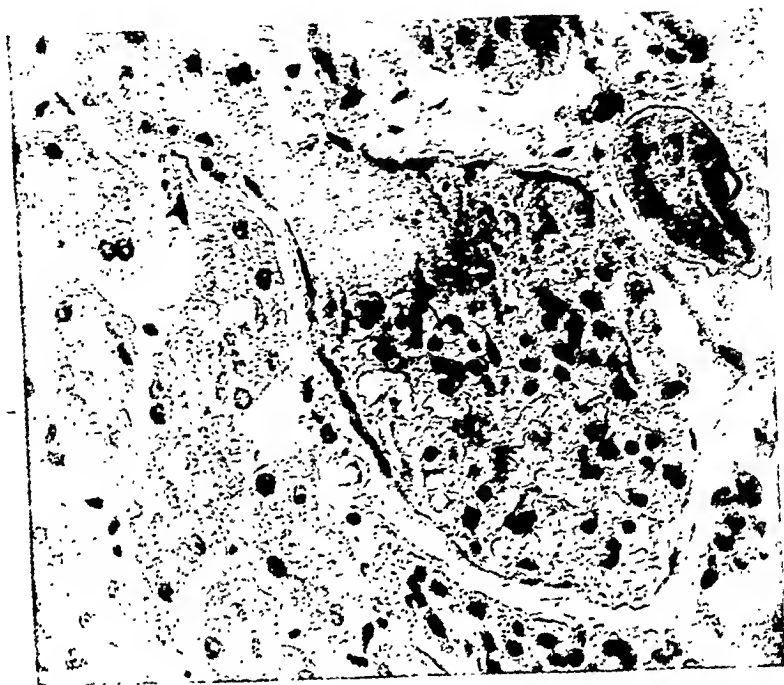


FIG. 1.

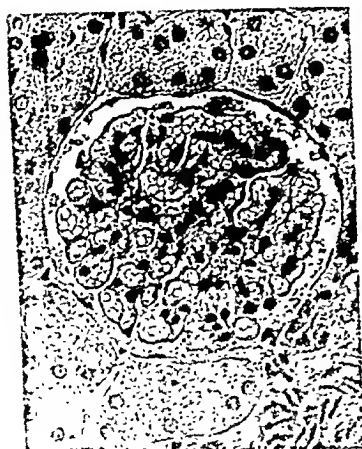


FIG. 2.



FIG. 3.

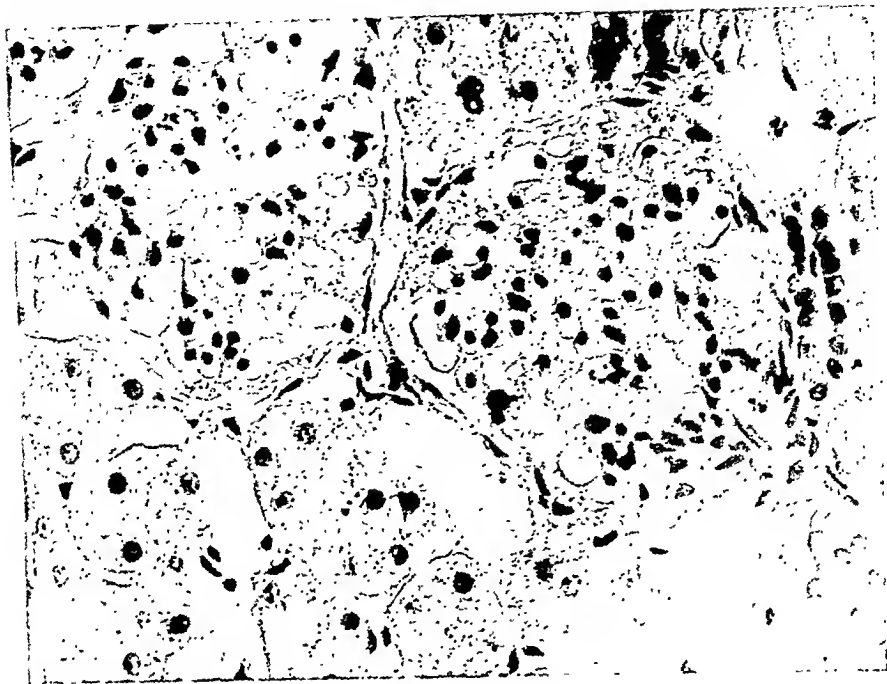


FIG. 4.

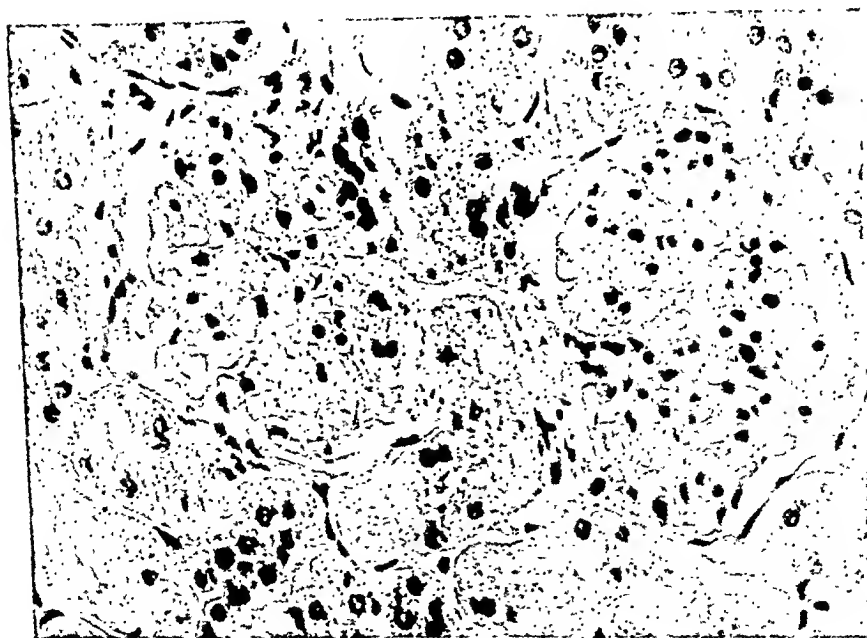


FIG. 5.

(Duval and Hibbard: Endotoxin from *Streptococcus carnosus*.)

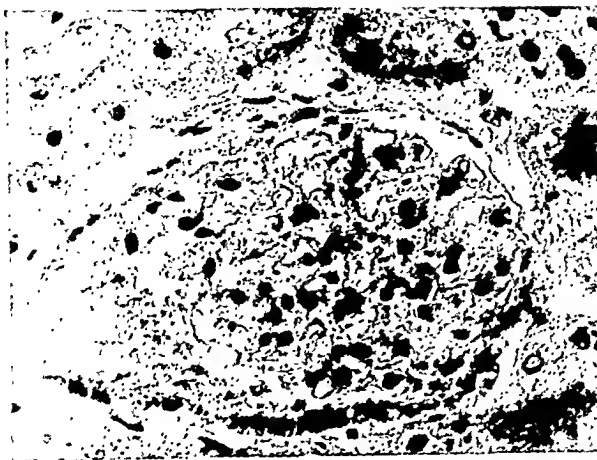


FIG. 6.

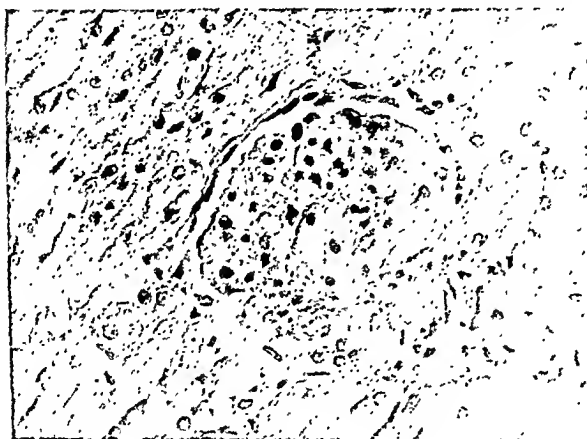


FIG. 7.

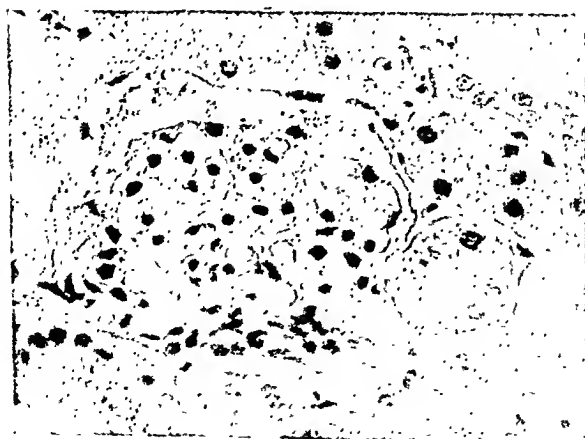


FIG. 8.

(Duval and Hibbard: Endotoxin from *Streptococcus scarlatina*.)

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Edited by

W. J. CROZIER

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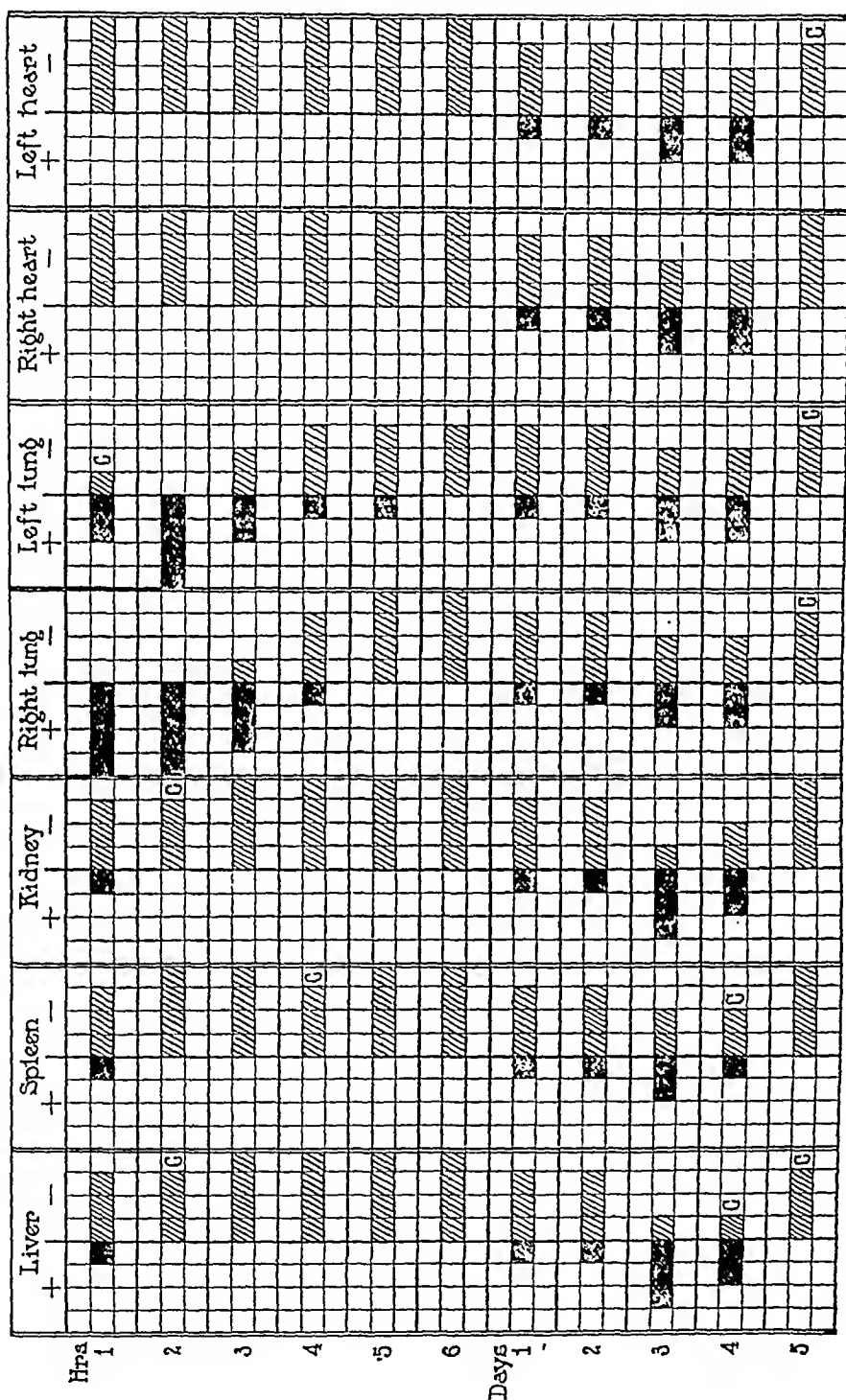
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TEXT-FIG. 1. Distribution of pneumococci in organs following spraying. The black squares indicate organs in which cultures showed pneumococci to be present, the cross-hatched squares represent organs in which cultures remained sterile.

SUSCEPTIBILITY OF RABBITS TO INFECTION BY THE INHALATION OF VIRULENT PNEUMOCOCCI.

By ERNEST G. STILLMAN, M.D., AND ARNOLD BRANCH, M.D.

(From the Hospital of The Rockefeller Institute for Medical Research.)

(Received for publication, June 18, 1926.)

In preceding papers (1, 2) it has been shown that following inhalation, virulent pneumococci generally disappear from the lungs of normal mice within a few hours, that infection rarely occurs, and that in only a few instances does death result from septicemia. It has further been shown that a high degree of active immunity may develop in mice following repeated inhalations of living pneumococci. However, mice are not suitable for tracing serologically the varying degrees of immunity developed by the repeated inhalations. Since rabbits may be easily and repeatedly bled, they were chosen for this work.

In the present paper are reported (1) the distribution of pneumococci in the organs of rabbits following spraying with pneumococci, (2) the occurrence of positive blood cultures, (3) the mortality of the exposed rabbits, and (4) the pathology of the pulmonary lesions in the rabbits which died. The immunity following upon the inhalation of pneumococci will be dealt with in a subsequent paper.

Method.

The rabbits were placed in a large spray chamber similar to that already described (2) and exposed to a spray of virulent Type I pneumococci. 150 cc. of an 18 hour broth culture was used for each spraying. The animals were exposed at 10 day intervals. Before each spraying, a sample of blood was obtained by bleeding the animal from the ear vein. At varying intervals after the spraying, blood cultures were taken from the ear vein. In the case of the animals which were killed and autopsied, broth cultures were made from both sides of the heart, and small pieces of the periphery of both lungs, and the kidney, liver, and spleen were placed directly in tubes of broth. Heart's blood cultures alone were made from the rabbits which died. All cultures were plated on blood agar for further identification. The animals killed with chloroform were immersed in a solution of lysol and opened with sterile instruments.

TABLE I.

Days on Which Positive Blood Cultures Were Obtained Following a Single Exposure to a Pneumococcus Spray.

Rabbit No.	Days..1	2	3	4	5	6	7	8	9	Course
1	c	c	—	—	—	+				Survived
2	—	+	+	—	—					"
3	+	+	+	+						Died 6th day
4	+	—	—	—	—	—				Survived
5	—	+	c	c		+				Died 7th day
6	—	+	+	c	c					" " "
7	—	—	—	c	+		—			Survived
8	+		c			—				"
9	—	—	+	+						Died 6th day
10	—	c	—	+						" 5th "
11	+	c	—	—	—					Survived
12	+	+	—		—	—	—	—	—	"
13	+	—	—		—	—	+	c	—	"
14	—	—	—		—	—	+	—	—	"
15	—	—	—		c	—	+	—	—	"

Blood cultures in 29 rabbits were negative.

A cross indicates a positive culture of pneumococcus, a dash a sterile culture, and "c" a contaminated culture.

TABLE II.

Fate of Rabbits after One to Ten Exposures to Inhalations of Pneumococci.*

No. of times exposed to virulent pneumococci	No. of rabbits exposed	No. dying	Pneumococcus recovered from heart's blood	Pneumococcus not recovered from heart's blood
1	231	86	80	6
2	111	9	8	1
3	98	11	9	2
4	71	4	2	2
5	50	1	—	1
6	39	1	—	1
7	34	—	—	—
8	31	—	—	—
9	31	—	—	—
10	27	—	—	—

* The discrepancy between the total numbers and the sum of the numbers in the individual groups is due to the fact that certain of the animals that survived were used for intraperitoneal tests or for other experiments.

Distribution of Pneumococci in the Organs.

In Text-fig. 1 is shown the distribution of pneumococci in the organs of 44 sprayed rabbits which were killed at intervals after spraying. It will be seen from the figures for the 24 rabbits killed in from 1 to 6 hours that pneumococci were generally recovered from the periphery of the lungs for the first 3 hours after the spraying but that later they rapidly disappeared. In only 1 rabbit were pneumococci recovered from the liver, spleen, and kidney. The heart's blood cultures from this animal remained sterile. Of the remaining 20 rabbits killed from 1 to 5 days following exposure, 6, or 30 per cent, showed a general pneumococcus septicemia. In 1 rabbit killed on the 3rd day, pneumococci were recovered only from the liver and kidney. This experiment shows how readily normal rabbits may become infected following inhalation of pneumococci. It further indicates that a transient pneumococcus septicemia may be present following spraying. In order to gain further knowledge of this point daily blood cultures were taken from the ear vein of a few rabbits.

Blood Cultures.

From Table I it is seen that following a first exposure positive blood cultures were obtained in 15 out of 44 rabbits. Of the 15 rabbits which developed a septicemia 5, or 33 per cent, continued to have a positive blood culture until death. The other 10 rabbits had merely a transient septicemia and survived. In some instances the septicemia could be detected during the first 24 hours but in others the organisms were first recovered as late as the 6th or 7th day.

Mortality of Rabbits Following Exposure to a Pneumococcus Spray.

Table II shows the number of normal rabbits which succumbed during the course of spraying with virulent pneumococci. From this table it is shown that out of 231 rabbits 80, or 38 per cent, died of a pneumococcus septicemia following their first exposure. Although some rabbits escaped infection at the first exposure, 7, or 6 per cent, died after the second spraying, and an occasional rabbit died after the third and fourth exposures. Of the 112 rabbits which died, the blood of 13, or 11 per cent, was sterile. These rabbits probably succumbed

ical evidence of an attempt at localization of the infection. The lungs of this rabbit which died on the 4th day following the first exposure showed an interstitial inflammation (3).

DISCUSSION.

From the foregoing experiments it appears that rabbits are even more susceptible than mice to infection with pneumococci following inhalation. For a period of 3 hours after spraying the organisms may be demonstrated at the periphery of the lungs. It is unfortunate that their subsequent course deeper into the lungs cannot be followed. But the great number of other organisms which normally inhabit the bronchi of rabbits, as Jones (4) has shown, renders cultural studies of the lungs difficult. Histological detection of pneumococci in the tissues is also unreliable when the organisms are present only in small numbers.

In all probability the majority of the organisms are destroyed either when they first localize or deeper in the lung tissue by the endothelial leucocytes or by polymorphonuclear leucocytes. In any case a few organisms occasionally filter through into the blood stream. Gaskell (5) believes that invasion of the blood stream by pneumococci probably always takes place in the early stages of an air-borne infection of the lung. The actual invasion of the blood stream must be much more common than is supposed. Not only are there probably few organisms free at any one time in the circulating blood but these few bacteria may even be within leucocytes and not in reality multiplying in the blood.

The occasional organisms which reach the blood are probably rapidly filtered out of the blood stream by the organs and locally destroyed. It is evident, however, that following the apparent disappearance of the pneumococci from the periphery of the lungs, they again appear, this time in the blood, and as a result may be recovered from all the organs. The point of this reinvasion is as yet uncertain.

Following the initial spraying with pneumococci the majority of susceptible rabbits die of an overwhelming septicemia without any attempt on the part of the body to localize the infection.

to suffocation from being enclosed in the spray boxes too long. This technical error was corrected in later experiments. The table also brings out the fact that the greatest number of deaths occurred after the first exposure. After each successive spraying the mortality tended to lessen until after the fourth exposure no animals succumbed. In other words, those rabbits which were most susceptible to pneumococcus died early. As will be shown in a subsequent paper, the surviving rabbits were those which not only had the greatest natural resistance but also had acquired a certain degree of immunity as a result of repeated exposures to pneumococcus.

The interval elapsing between the time of spraying and death from pneumococcus septicemia is shown in Table III. From this it is seen that the greatest number of animals died within the first 7 days fol-

TABLE III.

Number of Days Elapsing between Spraying and Death of Rabbits from Pneumococcus Septicemia.

	Days. .1	2	3	4	5	6	7	8	9	10
Spray I.....	3	12	16	8	14	12	8	5	1	1
" II.....	—	2	—	3	1	—	—	—	1	1
" III.....	2	—	2	—	—	1	2	2	—	—
" IV.....	—	—	1	—	—	1	—	—	—	—

lowing the initial exposure, although an occasional rabbit survived longer. The time of death of the rabbits which had survived the first exposure is much more diverse, and these rabbits have a tendency to die after a longer interval.

Pathology.

In no instance was there any gross evidence of pneumonia, although serous and serofibrinous pleurisy and serofibrinous pericarditis were common. A histological examination was made of the lungs of 77 rabbits which died of pneumococcus septicemia following spraying. The lungs appeared normal in 20. Congestion and pleurisy were noted in 7 instances, edema in 25, congestion in 38, and hemorrhagic extravasation into the alveoli in 19. In only 1 instance was there any histolog-

CONCLUSIONS.

1. Rabbits are very susceptible to infection by inhalation of Type I pneumococci.

2. When rabbits are exposed to a pneumococcus spray the bacteria readily penetrate into the lower respiratory tract. The pneumococci which reach the periphery of the lungs as a result of this procedure usually disappear within a few hours but a generalized and fatal septicemia frequently later appears. Pneumococci may then be recovered from the periphery of the kidney, liver, and spleen. In the animals which die pleurisy and pericarditis are common but pneumonia does not occur.

3. Rabbits may recover from pneumococcus septicemia.

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and Brown and Pearce (6) called attention to this point several years ago. As they very aptly expressed it, one must be able "to see beyond the reaction at the site of reinoculation." More recently the same question has been raised by other workers in this field (7-9).

During the past few years in the course of a series of experiments, some of which have been reported in other communications, we have had occasion to carry out a number of reinoculations in treated syphilitic rabbits. Keeping in mind the principle elaborated above, we directed our attention, in judging of the results of reinoculation, not alone to (a) the occurrence of a lesion at the site of reinoculation, but also to the possibility of (b) the establishment of infection without the production of a local lesion, to (c) the occurrence of dissemination of the virus under these conditions, and to (d) the production of a positive Wassermann reaction. The purpose of this communication is to bring together the results of reinoculation of treated rabbits studied from these points of view. The evidence afforded by this study indicates that it is possible successfully to reinoculate a treated rabbit without the production of any clinically detectible lesion at the site of reinoculation; furthermore, that the time at which treatment is begun and the method of reinoculation are of importance in bringing about this type of response to a second infection.

In this communication we shall deal only with the results in treated rabbits, reinoculated with homologous strains of *Treponema pallidum*. It is obvious that in untreated rabbits reinoculated with the same strains it would be a most difficult feat to demonstrate a successful reinoculation if such occurred without a lesion being produced at the second portal of entry.

In the first part of our report we shall discuss the results obtained when both first and subsequent inoculations were intratesticular, and in the second part we shall deal with the results obtained when the first inoculation was intratesticular and the second was made by inoculating a granulating wound produced on the back of the animal. The Nichols strain was used in all the experiments.

STUDIES IN EXPERIMENTAL SYPHILIS.

VI. ON VARIATIONS IN THE RESPONSE OF TREATED RABBITS TO REINOCULATION; AND ON CRYPTOGENETIC REINFECTION WITH SYPHILIS.*

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The decision in any given case as to whether or not a syphilitic animal, treated and then reinoculated, has acquired a second attack of syphilis, will, of necessity, depend upon the criteria of a successful reinfection. In the older experimental work attention was directed mainly, if not entirely, to the occurrence of a local lesion (chancre) at the site of reinoculation. If no such lesion developed in the reinoculated animal it was assumed that a second infection had not been established. It is at once apparent that this assumption is justified only if syphilitic infection is always accompanied by the occurrence of a primary lesion at the portal of entry. That syphilis may occur in human beings without the development of a chancre is well known (1). The same has been shown to hold true for monkeys (2) and for rabbits (3-5). In view of these now well established facts it is clear that, in determining in the reinoculated animal whether or not a second infection has been established, one must take into consideration the possibility that second infections may occur without the appearance of any local lesion at the portal of entry. Such second infections might be characterised by dissemination of the treponemata throughout the body and their lodgement in distant organs, with or without the production of lesions at the site of such lodgement. Neisser (2) admitted the possibility of such a result from reinoculation in his discussion of superinfection,

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to no infection at all. These six theoretical possibilities may be stated as follows:

Response A.—The production of a local lesion at the site of reinoculation accompanied by dissemination of the virus, in other words, a reaction which is identical with that of normal rabbits to syphilitic infection.

Response B.—The production of a local lesion at the site of reinoculation without evidence of dissemination of the virus.

Response C.—The persistence of virus at the site of reinoculation and dissemination of the virus but without the development of any local lesion.

Response D.—Dissemination of the virus without the production of a local lesion and without the persistence of virus at the site of reinoculation.

Response E.—Persistence of the virus at the site of reinoculation without dissemination and without the development of a local lesion.

Response F.—Absence of a local lesion and absence of virus at the reinoculation site together with no dissemination of the virus; in other words, a negative result.

It is, of course, questionable how far one may go in distinguishing Response D from Response C, since it is always conceivable that the virus may have been present at the site of reinoculation but have been overlooked due to insufficient examination. Negative results are notoriously misleading, but the theoretical possibilities exist, and it is of interest to see how closely the observed facts fit them, when reasonably diligent attempts are made to find the organisms at the site of reinoculation.

Of the six possible responses to reinoculation, examples of five were encountered in one or more rabbits in the group of twenty-three reported upon. The distribution of these rabbits among the five responses, or categories, as we shall call them, is shown in Table I, together with the duration of the infection as well as the duration of the initial orchitis at the time treatment was begun.

Consideration of Table I shows that in six of the entire series of twenty-three animals a characteristic local lesion developed at the site of reinoculation, followed by evidence of dissemination of the virus (Category A). The duration of the primary orchitis and the

*I. All Inoculations Intratesticular.**Experimental.*

First Inoculation.—In each animal only one testis was inoculated with virus emulsion.

Treatment.—Arsphenamine was administered intravenously at weekly intervals for a total of six doses. The individual dose was 10 mg. per kilo in each instance. Treatment was begun at varying intervals after inoculation, ranging from 41 to 291 days. Lymph node transfer was performed in all but one of the animals *after* treatment but *before* reinoculation.

Reinoculation.—Reinoculations were in some instances performed only once and in others twice, and always in the testis opposite to that originally inoculated. They were carried out 53 to 69 days after treatment was concluded. Lesions developing at the site of reinoculation were, of course, examined for the presence of treponemata. If no lesion developed the testis was excised, an emulsion of the organ was made with normal saline solution and examined for treponemata by the dark-field method. In four instances where this emulsion was negative it was inoculated intratesticularly into normal rabbits. In every instance the reinoculated testis was studied by one or both of these methods.

Dissemination of Virus.—The possibility of occurrence of dissemination of the virus was ascertained by clinical observation and by transfer of a popliteal lymph node to the testes of normal rabbits. This was carried out from 90 to 101 days after reinoculation and it was not omitted in any animal reported upon in this paper. The period of observation following reinoculation was at least 90 days and in many instances longer. As judged by the behavior of the controls this period affords ample opportunity for the development of syphilitic lesions at the site of reinoculation.

Wassermann Reaction.—The Wassermann reaction was performed on the blood serum of many of the rabbits at frequent intervals before and after reinoculation, according to the technique outlined in the preceding paper of this series. The reaction was negative in every instance prior to reinoculation.

Results.

We are able to report upon a total of twenty-three treated syphilitic rabbits reinoculated and studied as described above.

If it be assumed that reinfection may take place without the production of a local lesion at the site of reinoculation, but may be manifested by local infection without clinical phenomena, or by dissemination of the virus, then it is apparent that there are six theoretically possible responses to reinoculation, ranging from the production of a second infection which is entirely similar to the first,

duration of the infection at the time treatment was instituted averaged 18.7 and 45.8 days respectively. The Wassermann reaction of the two animals of this group in which it was performed became positive after reinoculation. None of these animals exhibited generalised lesions following reinoculation.

There were no instances in which a local lesion was produced at the site of reinoculation without dissemination of the virus (Category B). In our experience whenever a local lesion has been produced with the Nichols strain at the site of reinoculation, it is invariably accompanied by dissemination of treponemata and involvement of distant lymph nodes.

In two animals (Category C) no local lesion was produced but careful search revealed the presence of treponemata at the site of reinoculation and there was definite evidence of dissemination of the virus. The average duration of the primary orchitis and of the infection at the time treatment was instituted in these animals was 27.5 and 54.5 days respectively. Both animals developed positive blood Wassermann reactions after reinoculation.

In six animals (Category D) no local lesion developed following reinoculation nor was it possible by careful search to demonstrate the presence of treponemata *in situ*, nevertheless, there was evidence of dissemination of the virus in all of them and they all showed positive blood Wassermann reactions following reinoculation. The average duration of the primary orchitis and of the infection at the time treatment was begun was 23.5 and 48.8 days respectively.

We encountered one rabbit, No. 15 (Category E), in which no local lesion developed at the site of reinoculation and in which there was no evidence of dissemination of the virus as judged by lymph node transfer or the occurrence of generalised lesions. Careful search of the testis (left) in which the reinoculation was made showed no treponemata by dark-field examination, but the emulsion of testicular material was inoculated into two normal rabbits and both developed syphilis. It should be noted that this animal was originally inoculated in the right testis and a characteristic syphilitic inflammation of this organ developed, but up to the time treatment was instituted, namely, 193 days after the original inoculation and 167 days after the primary orchitis was manifest, no macroscopic lesions had appeared

TABLE I.

Incidence of Various Types of Response to Reinoculation.

Category	Lesion at site of reinoculation	Virus at reinoculation site without lesion	Dissemination of virus (node transfer)	Rabbit No.	Duration of orchitis at time of treatment	Duration of infection at time of treatment	Wassermann reaction after reinoculation
A	+	0	+		<i>days</i>	<i>days</i>	
				1	17	49	Not done
				2	22	48	" "
				3	16	48	" "
				4	15	48	" "
				5	16	41	++++
				6	26	41	++++
				Total 6	Average 18.7	Average 45.8	
B	+	0	0	None	—	—	
C	0	+	+	7	23	41	++++
				8	32	68	++++
				Total 2	Average 27.5	Average 54.5	
D	0	0	+	9	16	41	++
				10	16	61	++
				11	26	41	++++
				12	26	41	++++
				13	19	41	++++
				14	38	68	++++
				Total 6	Average 23.5	Average 48.8	
E	0	+	0	15	167	193	0
F	0	0	0	16	179	209	Not done
				17	196	209	" "
				18	272	291	" "
				19	27	56	0
				20	182	193	0
				21	174	193	0
				22	174	193	0
				23	171	193	0
				Total 8	Average 172	Average 192	

of the virus at the site of reinoculation and its dissemination to distant lymph nodes. Still others may show no virus at the reinoculation site but give evidence of its dissemination nevertheless. In general, it would appear that reinfection of treated syphilitic rabbits may take place without any local lesion, following intratesticular reinoculation, and that this type of response to a second inoculation is apt to occur when treatment is begun from 41 to 68 days after the first infection. It would appear to be almost constantly accompanied by the development of a positive Wassermann reaction in the blood. Animals responding in this manner to a second infection may conceivably be regarded as in part refractory. Occasionally, as exemplified by Rabbit 19, an animal may become apparently entirely refractory to a second infection early in the course of the infection and remain so even if treatment is begun before the 69th day of the infection.

The behavior of Rabbit 15 is of interest in that it indicates a high degree of resistance to a second infection, which, however, falls just short of being complete. This animal was treated late in the course of the disease (193 days) and when reinoculated gave no indication of dissemination of the virus, but the presence of the latter at the site of reinoculation could be demonstrated by animal inoculation. The animal, moreover, failed to exhibit a positive Wassermann reaction following reinoculation.

Where the treatment was postponed for 6 months or more there was, except in the one instance noted above, no evidence that a successful reinfection had taken place. This observation is in accord with those of Kolle (10) and Frei (11) and would indicate that the refractory state in such animals is probably complete.

The data presented in Table I would indicate that in general the type of response (A) to a second inoculation characterised by the development of the essential disease picture (local lesion and dissemination of the virus) is most apt to occur when the primary orchitis is interrupted relatively early (average 18.7 days) by treatment. If treatment is withheld until the orchitis is slightly farther advanced (average 24.5 days) the response tends to become that characterised by dissemination of the infection without the production of a local lesion (D and E). If treatment is withheld until a much later date

in the opposite testis (left), which was the one reinoculated. Furthermore, this animal never developed a positive Wassermann reaction after reinoculation. The evidence is very suggestive that the virus recovered by animal inoculation from the reinoculation site was that introduced at the time of reinoculation. It would appear that the animal had been rendered highly, although not completely refractory to a second infection.

There were eight rabbits in which no local lesion developed at the site of reinoculation and in which the virus could not be demonstrated at that site (Category F). Furthermore, the virus could not be recovered from the lymph nodes of any of these animals following reinoculation and in all of them the Wassermann reaction remained persistently negative. Of the eight animals in this group, there were four in which the reinoculated testis was emulsified and, in addition to being studied by dark-field examination, was inoculated intratesticularly into each of two normal rabbits. In every instance this inoculation proved to be negative. In the remaining four animals the examination of the reinoculated testis was confined to dark-field examination of an emulsion of the organ. In this group, then, no evidence could be adduced to show that any of the animals had been successfully reinoculated. The average duration of the infection in this group at the time treatment was begun was 192 days. All but one of the animals falling in this group were treated late in the course of the disease. The single exception, No. 19, received its first treatment on the 56th day of the infection.

Comment.

The foregoing results indicate that when rabbits with syphilitic orchitis are treated with arsphenamine comparatively early in the course of the disease, that is to say before the 69th day, and are subsequently reinoculated intratesticularly in the opposite testis with the homologous strain of treponemata, the response to the second inoculation will vary within stated limits. Some animals may show a response similar to that of normal animals and are, therefore, to be regarded as non-immune. Other animals may show no local lesion at the reinoculation site but, nevertheless, will present evidence of having been successfully reinoculated as determined by the persistence

place and after the wound heals a characteristic chancre develops, which can even be made to conform to a predetermined pattern. It seemed of interest to determine how a rabbit, first infected by intratesticular inoculation and treated at a time when it could be presumed to be refractory to a second intratesticular inoculation, would react to a second infection in which the virus was deposited upon a granulating wound on the back, in the manner previously described.

It is at once apparent that such an experiment might throw light upon the question of the extent to which the refractory state, which develops in syphilitic rabbits in the course of time, is a property of the animal body as a whole, that is to say, a general resistance, as against a local resistance only. There is in the literature some evidence to show that not all of the tissues of the rabbit share equally in the resistance toward a second infection which syphilitic rabbits unquestionably acquire.

Tomasczewski (14) found that if rabbits were inoculated in the cornea with syphilitic virus and a syphilitic keratitis developed, such rabbits, in most instances, could be successfully infected a second time by subscrotal inoculation as late as 128 days after the appearance of the keratitis. He also found that if the first infection were produced by subscrotal inoculation it was possible to produce a syphilitic keratitis in 76 per cent of his animals by inoculating the cornea from 39 to 105 days after the first inoculation. Apparently he reinoculated with an homologous strain of *treponemata*. Uhlenhuth and Mulzer (15) also succeeded, in three of four experiments, in obtaining second infections by intratesticular inoculation when the first infection was produced by corneal inoculation. In contradistinction to Tomasczewski, however, they were unable to obtain, in eleven of thirteen experiments, a second successful infection by the corneal route when the animals had been previously inoculated intratesticularly and a characteristic syphilitic orchitis had been produced. In two of their animals, however, they did succeed in producing a keratitis following testicular inoculation. The interval elapsing between first and second inoculations in their experiments ranged from 40 to 170 days. The two successful second infections were obtained when the second inoculation was carried out 40 and 61 days, respectively, after the first. These investigators were apparently using homologous strains for first and second inoculations.

Zinsser (16), largely on the basis of these experiments together with some of his own, has formulated the principle that the refractory state which develops in syphilitic rabbits is in reality a local phenomenon dependent upon previous infection at the site of reinoculation. Thus, his conception of syphilitic immunity is restricted even more than that of Kraus and Volk (17) who have suggested that it is a tissue immunity restricted to certain tissue groups and not shared to the same extent by all tissues.

(6 months or more), when the animal has had opportunity to heal the initial lesion by virtue of its own defensive mechanism, no infection occurs following intratesticular inoculation, or at most a local infection without local lesion and without dissemination of the virus. These facts point to a gradually developing resistance, the first manifestation of which is the ability of the animal to suppress the local reaction.

It may be objected that the recovery of the virus from distant lymph nodes of treated reinoculated animals in which no local lesion is produced at the reinoculation site, is not conclusive evidence of the success of reinoculation, since it is conceivable that the virus recovered from such nodes may be that of the first infection. This objection may be met with the statement that this phase of the experiment was controlled in all but one of the animals by removal of a node *after* treatment but *before* reinoculation, and transfer of the latter to normal animals. All such transfers proved to be negative. Furthermore, study of other animals similarly treated but *not* reinoculated showed that treatment was uniformly effectual in rendering the nodes non-infectious. Even if nodes were removed from such rabbits as late as 201 days after the last dose of arsphenamine they always failed to convey the infection to normal animals. Similar results were obtained by Nichols and Walker (12) with even fewer doses of arsphenamine than we have used. In addition, the occurrence of a positive blood Wassermann reaction following reinoculation, when previously it had become negative under treatment, offers additional support for the view that such animals were in truth reinoculated successfully and that the virus obtained from the lymph nodes did not originate from the first reinfection but represented that introduced at the time of the second inoculation.

II. Reinoculations on Wounds.

In a previous communication (13) we have called attention to the fact that a granulating wound (11 to 16 days old) on the back of a rabbit offers a particularly favorable site for the development of a chancre after inoculation with the Nichols strain of *T. pallidum*. When a testicular emulsion of the virus is allowed to drop on the exposed granulating surface of such a wound, infection readily takes

strain (Nichols) of treponemata was used for reinoculation. The virulence of the inoculum was controlled by inoculating five normal rabbits with wounds of the same age and prepared in the same way. Reinoculation was performed 248 to 290 days after the first inoculation. Wassermann reactions were performed at frequent intervals both before and after reinoculation. In addition a single popliteal node was removed from each test animal and from two of the virus controls 60 to 63 days after reinoculation; it was emulsified in normal saline solution and the entire emulsion inoculated into the testes of each of two normal rabbits. The test animals were observed for a period of 195 days following reinoculation before the experiment was terminated.

Results.

The results are shown in Table II.

In all of the animals the wounds healed at about the rate expected in uninoculated rabbits. In two of the test animals abscesses developed and these were incised and drained. No treponemata could be demonstrated in the exudate from these infected areas. In one animal (No. 32) a characteristic chancre developed after an incubation period of about 25 days. Treponemata could be demonstrated with ease in the serum obtained from this lesion. It is of interest that this chancre began to regress spontaneously 47 days after its appearance, long before there was any sign of spontaneous regression in the lesions which developed in the controls. There were never any signs of generalised syphilis in this rabbit but the Wassermann reaction became positive after reinoculation and the lymph node transfer was also positive. In no other rabbit did a syphilitic lesion appear at the site of reinoculation. It is true that several animals exhibited transient induration of the scar at about the time of complete healing, but serum from these areas did not show treponemata by dark-field examination and no lesions developed subsequently. In five rabbits, including the one in which the chancre developed, the Wassermann reaction became positive. Lymph node transfer was positive in three of these five animals. Altogether, in eight of the sixteen test animals lymph node transfer was positive and in only one of these did a syphilitic lesion develop at the site of inoculation. In none of the sixteen test animals did any generalised syphilitic lesions make their appearance.

In the five control animals a typical chancre appeared in each

Experimental.

Sixteen animals were inoculated intratesticularly with the Nichols strain of *T. pallidum* and the infection was permitted to run its course for a period of more than 5 months. All the animals were then treated with arsphenamine intravenously in exactly the same manner as in the preceding experiment. Treatment was begun 159 to 201 days after inoculation, the average being 169 days.

TABLE II.
Reinoculation of Treated Rabbits.

Rabbit No.	Wassermann reaction before reinoculation	Result of reinoculation			
		Lesion at site of reinoculation	Generalised lesions	Wassermann reaction	Node transfer
24	Negative	None	None	Negative	Negative
25	"	"	"	"	Positive
26	"	"	"	"	Negative
27	"	"	"	"	Positive
28	"	"	"	"	Negative
29	"	"	"	"	"
30	"	"	"	Positive	"
31	"	Abscess	"	"	Positive
32	"	Chancre	"	"	"
33	"	None	"	Negative	Negative
34	"	Abscess	"	Positive	Positive
35	"	None	"	Negative	"
36	"	"	"	"	"
37	"	"	"	"	Negative
38	"	"	"	"	Positive
39	"	"	"	Positive	Negative
Virus controls					
40	"	Chancre	"	"	Not done
41	"	"	"	"	" "
42	"	"	"	"	Positive
43	"	"	"	"	Not done
44	"	"	"	"	Positive

89 days after the last dose of arsphenamine was administered the animals were reinoculated on the back. This was accomplished by first excising an elliptical area of skin, under ether anesthesia, exposing the subcutaneous tissue. The wounds were not dressed in any way and 14 days after operation, when granulation tissue was well established, the crusts were removed with as little trauma as possible and 2 drops of a testicular emulsion, rich in actively motile treponemata, were allowed to drop on the exposed granulating surface. The same

treatment carried out in another group of syphilitic rabbits at a comparable period in the course of the disease (Category F, Table I) had failed to bring about cure in any, since they were all refractory to a second inoculation. This explanation does not seem logical and has the disadvantage in that it gives no clue as to why identical treatment should fail completely in one experiment and be 50 per cent effective in another, other things being equal. Nevertheless, if it is correct, then the experiment necessarily indicates that treatment late in the course of the infection in rabbits will apparently cure at least half of the animals.

A second and perhaps more plausible explanation which is suggested is that the resistant state which develops in syphilitic rabbits as a result of their infection is not absolute but is capable of being broken down in part by resort to a method of inoculation which appears to be particularly favorable to the inciting agent. It is possible that this breaking down of the animal's resistance is accomplished through failure of the granulation tissue of these healing wounds to share in the resistant state to the extent as does the testis for example, whether or not the latter organ has previously been the seat of a syphilitic inflammatory process. Nevertheless it would seem that the newly formed granulation tissue does share to some extent in the immune process since half of the reinoculated animals were, so far as we could judge, not reinfected, and since in only one of the remaining half, which apparently were successfully reinfected, did a characteristic chancre develop in the wound.

It is entirely possible that the tissues in these granulating wounds in reality did possess a high degree of resistance to a second infection but that the trauma incidental to removal of the crust of the wound preparatory to inoculation resulted in the opening up of lymph channels and thus facilitated migration of the treponemata and invasion of the blood stream before any local defensive process could effect a complete destruction of all the treponemata. If this is the correct explanation of the apparently successful reinfection of half of the animals, it would speak against a humoral immune mechanism in syphilitic rabbits, at least of great magnitude.

It is necessary to consider also the possibility that there is no real resistance to reinfection in these granulating wounds in late treated

instance. These went through the ordinary course of development such as we have described previously, and finally healed spontaneously. In all five the Wassermann reaction became positive and lymph node transfer in the two instances in which it was done was positive. No generalised lesions were noted in the controls during the period of observation.

Comment.

It is clear that in but one of these sixteen treated and reinoculated rabbits did there develop a characteristic chancre following reinoculation upon a granulating wound. The remaining fifteen failed to show anything at the site of reinoculation which might be considered as syphilitic, and yet, in seven of these fifteen, lymph node transfer was positive, indicating that the animals were syphilitic at the time transfer was performed. For reasons outlined above we are inclined to the view that the virus obtained from these lymph nodes was that introduced at the time of reinoculation, and we interpret the experiment as indicating that at least half of the test animals had been successfully reinfected by inoculating a granulating wound on the back although in only one did a syphilitic lesion develop at the portal of entry.

The high incidence of successful reinoculations obtained in late treated syphilitic rabbits when the virus was deposited upon a granulating wound on the back (50 per cent), as contrasted with the comparatively low incidence of successful reinoculations obtained when the virus was injected intratesticularly, calls for explanation. One possibility that has to be taken into consideration involves the contention of Neisser that acquired immunity to syphilis is dependent upon the persistence of active foci of syphilitic infection somewhere in the body of the host, and furthermore that the response of a treated animal to reinoculation can be taken as an index of cure. In previous publications we have discussed this theory and need not therefore consider its validity at this point. Assuming it to be true, the results outlined in the second experiment are susceptible of but one interpretation, namely, that although treatment was begun late in the disease half of the animals were cured while half were not. On the same basis it would have to be concluded that the same mode of

of treponemata may be produced in a large proportion of treated rabbits without the development of any syphilitic lesion at the site of reinoculation.

The results of the experiment recorded in Table II, when contrasted with those outlined in Table I, seem to throw additional light upon the question of the advisability of using the reinoculation method as a criterion of the cure of syphilis. Our previous experiments, in conformity with those of Kolle and of Frei, have shown that when rabbits are infected by the intratesticular route and are treated with arsphenamine late in the course of the infection (181 to 291 days) they are almost uniformly (over 90 per cent) refractory to a second infection introduced in the testis. According to the reinoculation test these animals were not cured. On the other hand, in a group of sixteen animals originally inoculated in the testis and treated 159 to 201 days after infection with the same drug and the same dosage, successful reinoculations (with the same criteria) were apparently accomplished in eight, or half of the animals, when the second inoculation was made upon a granulating wound on the back of the animal. Using the reinoculation test as a criterion of cure one would have to assume that half of these animals had been cured and the other half had not. If comparable groups of rabbits treated in exactly the same manner can give such different results when reinoculated in different ways (90 per cent or more against 50 per cent), how is it possible in such experiments to draw any deductions, from the result of the reinoculation, as to the persistence or absence of the first infection in the body of the host, that is to say, cure or failure to cure? Who is to say which method of reinoculation is the one by which to judge? One can scarcely escape the conclusion that, in view of the variable results given by different methods of reinoculation, such a procedure cannot be accepted as a valid method for determining the possibility of cure of syphilitic infection.

It is perhaps not amiss to point out that the experiments outlined above may have clinical significance. If it is true that under certain conditions a treated syphilitic rabbit can be successfully reinfected without the occurrence of a local lesion at the site of reinoculation, but, nevertheless, with the development of a positive Wassermann reaction in the blood and with dissemination of the treponemata,

syphilitic rabbits, that is to say no active local defensive mechanism. It may be that all that is being observed is an instance of acquired inability of tissues to react in the customary manner to syphilitic virus, that is, an indifference or state of anergy in the sense that Neisser used the term. If such is the correct view then we must consider the possibility of a humoral factor in acquired resistance to syphilis since at least half of the test animals were apparently refractory to a second infection. Regardless of whether one considers this inability to produce a characteristic chancre by inoculating granulating wounds of late treated syphilitic rabbits as evidence of tissue indifference or anergy, or as evidence of the existence of a local active defensive mechanism more or less complete, it must be admitted that the acquired property of the granulation tissue not to react is not dependent upon a preexisting local syphilitic infection. By no stretch of the imagination can one conceive of such granulation tissue having been previously infected with syphilis since the wounds were made after the animals had been thoroughly treated. The more or less refractory state of the granulating wound in the immune animal may be dependent upon either a humoral defensive mechanism, or a mechanism limited to cells, or a combination of both factors. Whichever factor is operative, the experiment shows clearly that the resistant state which develops in rabbits during the course of syphilitic infection is in part at least conferred upon newly formed granulation tissue (with all that that term implies) and suggests that the immune mechanism, if cellular, is capable of being inherited by, or imparted to, newly formed cells. The experiment suggests also that a method is at hand for successfully reinfecting syphilitic rabbits which from past experience one would be justified in assuming to be refractory to an intratesticular inoculation.

Whatever is the correct explanation of the facts that have been observed, it seems clear that in considering the results of reinoculation of treated syphilitic rabbits the experiments cited above would indicate that it is essential to bear in mind that the time at which treatment is begun and the mode of reinoculation are important factors in determining the character of the response to a second infection. Furthermore the evidence is strong that under the conditions of the experiments a second syphilitic infection with the homologous strain

be recovered from the reinoculation site although no local lesion is produced there and no dissemination of the virus can be shown to take place. An occasional animal treated before the 69th day of the disease remains completely refractory to a second infection.

When treatment is postponed to 6 months or more after the original inoculation, reinfection carried out by intratesticular injection is almost always impossible and such animals appear to be entirely refractory. However, if rabbits treated late are reinoculated with the homologous strain by depositing the virus upon a granulating wound on the back, successful reinfections can be accomplished in at least 50 per cent of the test animals. The resistance which develops in rabbits in the course of syphilitic infection is, then, not absolute but relative.

It is pointed out that these results cast discredit upon the validity of the reinoculation method as a test of cure in syphilitic infection. It is also suggested upon the basis of these experiments, that the subsequent occurrence of a positive Wassermann reaction in patients with early syphilis in whom the Wassermann reaction has become negative under treatment may not always represent a relapse in the the disease but possibly in some instances a new infection without clinical signs.

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then one may well raise the question whether such a phenomenon ever occurs in human beings. Every syphilologist is familiar with the patient who comes under treatment relatively early, who is systematically treated, whose Wassermann reaction becomes negative, and who subsequently, months or years after the treatment has ceased, suddenly shows a completely positive Wassermann reaction in the blood without any signs of syphilis anywhere. Such cases have in the past been regarded usually as instances of a serological relapse, and, by inference, of a failure to cure. In the light of our experiments it may well be that such cases in some instances represent examples of a second infection without signs of syphilis, rather than instances of Wassermann reaction recurrence, and that this conceivable modification of the usual response to syphilitic infection, which they exhibit, is the result of the influence of the first infection. It is universally admitted that second attacks of syphilis with all the characteristic phenomena of a first infection do occur, although rarely. It is not such a far cry to imagine that second infections may take place in treated individuals, with no more evidence of their existence than the development of a positive Wassermann reaction. The importance of deciding whether such cases are really instances of a new infection, rather than examples of a relapse of the old, must be manifest so long as reinfection in humans is regarded as adequate evidence of cure. The clinical differentiation of a case of Wassermann reaction relapse from that of a reinfection without manifest signs but attended by the development of a positive Wassermann reaction, assuming that it occurs in man, would probably be an almost insurmountable task.

SUMMARY.

Syphilitic rabbits inoculated intratesticularly and treated with arsphenamine before the 69th day of the disease, when reinoculated with the same strain of treponemata and in a manner identical with that of the first inoculation, are capable of responding to the infection in at least five different ways. In addition to exhibiting a local lesion at the site of reinoculation, accompanied by dissemination of the virus, they may show no local lesion at all but present evidence of dissemination of the virus together with the development of a positive Wassermann reaction. In some instances the virus may

active against this organism. These lytic filtrates were selected from several isolated by us at different times from the stools of mice recovering from experimental mouse typhoid, because they were typical of the variations in the character of lytic activity observed in these filtrates.¹

The overgrowth which was present in the cultures after 24 hours of incubation at 37°C. was streaked out on agar plates. Single colonies appearing on the plates were repeatedly transferred to fresh agar (each time from a single colony) in order to eliminate all traces of lytic principle. After seven successive transfers, 24 hours apart, each of the cultures was returned to broth. They proved to be free from lytic principle when filtered and tested against the original susceptible stock culture of M. T. II. Broth cultures themselves were tested at the same time for their susceptibility to lysis by each of the lytic filtrates in turn. As the results recorded in Protocol I demonstrate, each culture obtained from the overgrowth was resistant to lysis by the lytic agent used in its production, but only one of the cultures was resistant also to the activity of the second lytic agent.

Protocol I.

Identification of Resistants Isolated from Lysed M. T. II Culture.

Lytic agents used to obtain resistants	Character of growth in broth	Agglutinability with stock M. T. II serum 1:2000	Resistance or susceptibility to lysis	
			Resistant W-Little R ₇ *	Resistant W-178 R ₁ *
W-Little.....	Agglutinated	Positive	Resistant	Resistant
W-178.....	Diffuse	Positive	Susceptible	Resistant

* The legend R₇ added to the name of each culture indicates that it represents a resistant (R) variant of the seventh (7) passage on agar.

The identity of these cultures with the parent culture of M. T. II was checked by the agglutination test, in which a stock anti-M. T. II serum diluted 1:2000 was used as agglutinin.

It was observed that in some cases the overgrowth appeared diffuse; in others it was spontaneously agglutinated. Since this difference in the appearance of overgrowth has been stressed in the literature (6, 7) we have carried out all the experiments with Strains W-178 and

¹ We are greatly indebted to Dr. Ida Pritchett for placing at our disposal the greater part of the animals used for isolation of lytic agents. In passing, it should be recorded that the lytic filtrates yielded by the examination of over 300 mice were, for the most part, active only against *B. dysenteriz*. Next in frequency were those active against *B. enteritidis* Gaertner, and least frequently those active against the infecting organism M. T. II.

STUDIES ON THE BACTERIOPHAGE OF D'HERELLE.

VI. ON THE VIRULENCE OF THE OVERGROWTH IN THE LYSSED CULTURES OF *BACILLUS PESTIS CAVIÆ* (M. T. II).

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When susceptible bacteria are grown on broth in the presence of a suitable lytic agent, the initial turbidity of the culture gradually disappears because of the lysis of the bacteria. In exceptional cases the lysis may be complete and permanent, resulting in sterilization of the culture. But more often a few bacteria fail to undergo lysis and multiply in spite of the presence of lytic agent in the medium, thus giving rise to more or less profuse overgrowth of resistants, following a temporary clearing of the culture (1).

In the case of *B. coli*, Bordet and Ciuca (2) found that the overgrowth differs from the original culture, in addition to its resistance to lysis, by its active motility; and Gratia (3) found further that the overgrowth is more highly virulent. The heightened virulence of bacteria resistant to lysis was observed also by other investigators and has been suggested as the source of the failures of bacteriophage to influence favorably the course of infection in certain instances (4-7).

In view of the fact that the virulence of a strain M. T. II of *B. pestis caviæ* was carefully established during several years of study of experimental mouse typhoid at The Rockefeller Institute (8-10), we thought that a lysis-resistant overgrowth of this culture would offer exceptional material for comparison of its virulence with that of the original culture.

Isolation of Resistants.

In order to obtain pure strains of resistant bacteria, the stock culture M. T. II was grown in broth for 24 hours, in the presence of each of the two lytic filtrates

from an 18 hour broth subculture from the eleventh generation of a resistant W-Little R₁₁ on agar. The mice of the third group (Protocol II, C) each received 0.5 cc. of broth containing about 3,000,000 bacteria of an 18 hour broth subculture of W-178 R₁₁. Each animal was placed in an individual jar, its ordinary ration was supplied daily, and the litter was changed three times per week. No symptoms were noted until the 4th or 5th day after the feeding of bacteria, when certain animals in Group A appeared ill, and from the 6th day deaths began to occur, as indicated on Protocol II. The experiment was interrupted on the 15th day, after no deaths had occurred among the animals for 3 days. At this time all the animals receiving resistant bacteria (Groups B and C) were living, whereas only eight animals out of twenty-five (about 32 per cent) survived the feeding of the original M. T. II.

*Susceptibility to Infection by B. pestis caviæ (M. T. II) of Mice
Surviving the Feeding of Resistants.*

In the next two experiments the resistants of the thirteenth and fourteenth generations on agar were used. The method was the same as in the first experiment and the results may be summarized by stating that the mortality of controls (corresponding to Group A on Protocol II) was 62 and 66 per cent respectively, whereas all the animals but two of those fed the resistants remained alive. That these two deaths did not arise from mouse typhoid infection is rendered probable by the fact that at the autopsy the blood and internal organs were found to be free from *B. pestis caviæ*.

Since the failure of resistants to infect mice was contrary to what was expected on the basis of earlier reports in the literature (1-5, 11), it was thought advisable to ascertain the susceptibility of the surviving mice in the above experiments to infection with the parent culture M. T. II; if these animals prove susceptible to subsequent infection with M. T. II, their earlier resistance to W-Little R₁₄ and W-178 R₁₄ respectively can be ascribed to a change in virulence in these cultures.

Accordingly, twenty mice each of those (twenty-five) surviving feeding with W-Little R₁₄ and W-178 R₁₄ respectively (corresponding to Groups B and C on Protocol II) were divided into two groups. The first subgroup of ten mice from each group received a suspension of an 18 hour broth culture of virulent M. T. II by mouth, the other by intraperitoneal injection. Similarly, half of twenty normal mice were given the same bacterial suspension by mouth, and the other half by intraperitoneal injection. The animals were cared for exactly as in the earlier ex-

W-Little as representing this difference in growth. In order to avoid spontaneous return of susceptibility to lysis, the cultures were grown on agar and transplanted only as often as the experiments required. Whenever resistant bacteria were needed for the test, a subculture from agar was made into broth and used as such after 18 hours of incubation at 37°C.

Virulence of the Resistant by Feeding.

After having carried the resistant strains W-Little R₇ and W-178 R₇ for four more generations on agar (eleven in all) we attempted to determine their virulence as compared with that of the original strain of *B. pestis caviæ*.

Protocol II.

Virulence of Resistant by Feeding.

Bacteria fed		Group A (control) Stock M. T. II	Group B W-Little R ₁₁	Group C W-178 R ₁₁
No. of bacteria given.....		3,000,000	3,000,00	3,000,000
No. of mice surviving by days	5th day	25	25	25
	6th "	23	25	25
	7th "	19	25	25
	8th "	15	25	25
	9th "	12	25	25
	10th "	10	25	25
	11th "	9	25	25
	12th "	8	25	25
	13th "	8	25	25
	14th "	8	25	25

The animals used for these experiments came from special stock kept at The Rockefeller Institute, and known to have been free from spontaneous mouse typhoid for a period of years. It was hoped that these animals would show no heightened resistance to the infection, and would present an ideal object for the study to be undertaken.

75 mice of approximately 25 gm. weight each were divided into three equal groups. To each mouse of the first group (Protocol II, A) was given, by means of a stomach tube, 0.5 cc. of an 18 hour broth culture of *B. pestis caviæ* (M. T. II), diluted to contain about 3,000,000 bacteria in each dose. The mice of the second group (Protocol II, B) each received about 3,000,000 bacteria (in 0.5 cc. volume)

only in their power to resist lysis by homologous lytic agents, thus establishing their identity with the material fed to the mice. In other experiments of similar nature, the intestinal contents yielded an occasional colony susceptible to lysis; the bulk of bacteria fed to the mice seemed, however, to have remained in the intestinal contents without becoming susceptible to lysis, and without entering into the blood for at least 14 days. If this be proven to be the case with larger experimental material, it would suggest the loss of invasive power by the resistants.

Virulence of Resistants by Intraperitoneal Injection.

The fact that mice surviving the feeding of resistants were still susceptible to subsequent infection by the virulent parent M. T. II strain, together with the finding that the resistants lack invasive power, would indicate that their failure to kill animals in earlier experiments was due to this change in virulence, as suspected. However, we thought it of interest to inquire whether this change in virulence was limited to their loss of invasive power only, or to some more radical change. For this reason we compared their power to infect mice by parenteral route with that of the parent strain M. T. II. Accordingly, three series of fifteen mice each were given, by intraperitoneal injection, varying doses of the original M. T. II, W-Little R₁₄, and W-178 R₁₄ respectively, as indicated on Protocol IV, and the time of death of each mouse was noted. It will be seen from this protocol that whereas all the mice receiving the original M. T. II injection were dead before the expiration of 5 days (after injection), only three mice out of a total of thirty animals injected with resistants were dead up to the 10th day, when the experiment was terminated. We are inclined to attribute the death of these three mice to other causes rather than to the infection, particularly since in two subsequent experiments analogous to the one just described—except that the dose of bacteria was doubled and trebled respectively—only one mouse died, out of a total of 60 receiving resistant bacteria intraperitoneally, whereas the mortality of mice receiving the original M. T. II culture was invariably 100 per cent. These experiments, in our opinion, indicate that at least in the case of *B. pestis caviæ* isolation of cultures resistant to lysis by bacteriophage results in obtaining an avirulent strain of this organism.

periments, except that those injected intraperitoneally were placed in larger containers—five mice in each. Protocol III illustrates the results obtained.

The remaining eight mice surviving the feeding of resistants² were killed and examined for signs of invasion by the bacteria which had been fed to them. As in the case of two mice of this series which died

Protocol III.

Susceptibility of Surviving Mice to Infection by M. T. II.

No. of mice		Mice surviving the feeding of resistants 15 days previously				Normal controls	
		Survivals of Group B (fed W-Little R ₁₀)		Survivals of Group C (fed W-178 R ₁₀)			
		10	10	10	10	10	10
Mode of infection		By mouth	Intraperi- toneal	By mouth	Intraperi- toneal	By mouth	Intraperi- toneal
No. of virulent bacteria given (about).		5,000,000	2,000,000	5,000,000	2,000,000	5,000,000	2,000,000
No. of deaths per day	1st day		3		4		3
	2nd "		3		3		6
	3rd "		1		3		1
	4th "		2				
	5th "		1			1	
	6th "			1		2	
	7th "	2		3			
	8th "	1				1	
	9th "	3		1		2	
	10th "						
	11th "	1					
	12th "			1		1	
	13th "						
	14th "						
No. of survivals		3	0	4	0	3	0

earlier, the internal organs of these mice were sterile, but the intestinal contents showed the presence of bacteria resembling the parent strain M. T. II in their lack of ability to ferment lactose, in the production of H₂S, and in their immunologic properties. They differed

² Twenty mice of each series of twenty-five were used in the preceding experiment, and one animal in each group died from intercurrent cause, thus leaving four mice in each group (see experiment Protocol II).

Protocol V.
Virulence of the Reverted W-Little R₁₄ by Intraperitoneal Injection.

	A			B			C			
	Original culture of M. T. II			W-Little R ₁₄ (resistant variant)						
	Diffuse			Grown for 18 hrs. on broth			After 20 daily passages on broth			
	Susceptible			Agglutinated			Diffuse			
	Not susceptible			Susceptible						
Character of growth on broth.....										
Susceptibility to lysis by lytic agent W-Little										
No. of bacteria given intraperitoneally*										
No. of mice injected										
No. of mice surviving after 10 days (virulence).....										
Time of death after injection in days	10									
	9									
	8									
	7									
	6									
	5									
	4									
	3									
	2									
	1									
0										

* These counts were obtained by plating samples of suspensions of bacteria immediately after injection.

The Loss of Virulence by the Resis'ant Subcultures of M. T. II as Tested by Intraperitoneal Injection.

No. of bacteria given intraperitoneally.....	Original M. T. II (susceptible to lysis)				W-Little R _u (resistant to lysis)				W-178 R _u (resistant to lysis)			
	777,000	1,900,000	7,000,000		735,000	2,300,000	7,150,000	700,000	2,167,000	6,800,000		
No. of mice injected.....	1 2 3 4 5	1 2 3 4 5	1 2 3 4 5		1 2 3 4 5	1 2 3 4 5	1 2 3 4 5	1 2 3 4 5	1 2 3 4 5	1 2 3 4 5		
No. of mice surviving after 10 days..	None	None	None		All	All	Four	Four	All	Four		
Time of death after injection, in days	10th day	9th "	8th "	7th "	6th "	5th "	4th "	3rd "	2nd "	1st "	0	

* These figures show the bacterial count on agar plates poured immediately after injection of animals.

Virulence of W-Little R₁₄ after Its Recovery of Susceptibility to Lysis.

It is well known that bacteria isolated from the overgrowth of lysed cultures usually revert to the original type and become susceptible to lysis after a more or less prolonged cultivation on media free from lytic agent. Since the preceding experiments indicated that there may exist an interdependence between the lack of susceptibility to lysis and the loss of virulence in the culture of *B. pestis caviar*, it seemed desirable to find out whether the return of susceptibility to lysis will be followed by a return of virulence in such cultures.

For this purpose the fourteenth generation of a resistant strain (W-Little R₁₄) was subcultured from agar into broth, and from the latter daily transfers to broth were continued for 20 days, after which the culture thus obtained was tested for its susceptibility to lysis, as well as for its virulence to mice, by the intraperitoneal route. For the sake of comparison parallel tests were made with the culture of the original M. T. II, as well as with an 18 hour broth culture obtained by inoculating into sterile broth a small loop from the old agar slant W-Little R₁₄, which presumably retained its resistance to lysis by the phage W-Little. The procedure followed in this experiment was entirely analogous to that of the preceding experiment (Protocol IV), except that in addition to other tests, each culture was subjected to a control test of its susceptibility to lysis. The results of the virulence test are given in Protocol V. Similarly, the twentieth passage in broth from W-Little R₁₄ proved capable of killing mice when given by mouth (Protocol VI).

It is evident, then, that when full reversion to susceptibility to lysis, after prolonged cultivation in broth and in the absence of bacteriophage, has taken place, the resistant strain W-Little R₁₄, which remains avirulent and resistant to lysis on the first subculture in broth (Protocols V, B and VI, B), tends to become as virulent as the parent culture of M. T. II, from which it originally was derived.³

In order to ascertain more closely the relationship between the return of virulence and the susceptibility to lysis of the culture, the experiment was repeated and the test of susceptibility, as well as of virulence, was made daily.

³ It should be noted incidentally that simultaneously with these changes in virulence and susceptibility to lysis, the appearance of growth on broth changed from being agglutinated in the first subculture from agar to the diffuse growth characteristic of the parent culture M. T. II.

Protocol VII.

Respective Rate of Recovery of Virulence and of Susceptibility to Lysis by the Resistant Subcultures of W-Little R₁₄.

No. of passages on broth,	First	Second	Third	Fourth	Fifth	Sixth	Seventh	Eighth	Ninth	Tenth
Susceptibility to lysis by W-Little phage.....	Resistant	Resistant	Resistant	Resistant	Resistant	Susceptible but quickly overgrown	Susceptible but quickly overgrown	Susceptible but overgrown	Susceptible	Susceptible
No. of bacteria given intraperitoneally*	5,100,000	4,750,000	6,200,000	5,700,000	5,200,000	6,300,000	4,900,000	5,300,000	5,000,000	4,700,000
No. of mice.....	1 2 3 4 5	1 2 3 4 5	1 2 3 4 5	1 2 3 4 5	1 2 3 4 5	1 2 3 4 5	1 2 3 4 5	1 2 3 4 5	1 2 3 4 5	1 2 3 4 5
14 days										
13 "										
12 "										
11 "										
10 "										
9 "										
8 "										
7 "										
6 "										
5 "										
4 "										
3 "										
2 "										
1 "										

* The numbers recorded above were obtained by plating suspensions immediately after injection.

For this purpose we returned to the agar culture W-Little R₁₄ (kept on ice all the time), and made a subculture from it into broth. After 18 hours of incubation at 37°C. the resulting broth culture was tested for its virulence and susceptibility to lysis. A little later on the same day, a second subculture in broth was made from the first to be tested next day (after 18 hours of growth) and so on for several days (Protocol VII).

Only the intraperitoneal method of injection was used for testing virulence in this experiment, as it was deemed more convenient, both because of simplicity and the shorter incubation period. When it was observed that the animals died regularly, at a rate approximating that of the mortality of mice injected with the original M. T. II culture, the experiment was interrupted. Results of this experiment indicate that on the sixth transfer in broth the resistant culture W-Little R₁₄ became susceptible to lysis, and at the same time it regained its pathogenicity.

Non-Reversion to Susceptibility of W-178 R₁₄.

As stated in the early part of this paper, resistant strains obtained by us could be roughly divided into two groups, judging by the macroscopic appearance of their respective growth in broth. Since it has been observed further that resistants secured by means of the phage W-Little are susceptible to lysis by the phage W-178 (but not *vice versa*, see Protocol I), we undertook to ascertain whether resistants obtained by the action of the latter "stronger" phage would also undergo reversion to susceptibility and recover virulence if grown in the absence of phage. However, all attempts in this direction have thus far been unsuccessful. The cultures have remained resistant to lysis, and when tested for virulence, after nearly 200 successive passages in broth over a period of 10 months, mice survived an injection of 3,000,000 bacteria intraperitoneally. Throughout the period resistant bacteria exhibited their original characteristics as regards fermentation and antigenic properties, and were found to be free from bacteriophage (not "lysogenic"). Occasionally, when grown on agar plates with a corresponding bacteriophage, some of these cultures gave rise to a few "pale" plaques which were quickly overgrown by resistants. In such cases corresponding broth cultures have, on occasion, shown a slight increase in the titer of the phage, but at

to grow them in the presence of an antibacteriophage serum, in the hope that we might thus induce a reversion.

In these experiments a resistant strain, isolated by means of a phage P.I.D. and carried for several months without reversion, was employed. Starting from an agar slant culture of this strain P.I.D.-R₁₅, we have made two sets of daily transfers, the first into plain broth and the second into broth containing 0.02 cc. of antibacteriophage serum for each cc. of the medium. From time to time cultures of resistants thus obtained were tested for their susceptibility to lysis in broth as well as in agar. This procedure was continued for a month without leading to reversion, as illustrated on Protocol VIII.

DISCUSSION.

According to the original conception of d'Hérelle and his collaborators, the production of resistants is the result of an increase in the resistance of bacteria against the invasion of bacteriophage. They consider this process analogous to the development of active immunity in higher forms, after exposure to infection (19). In accordance with this theory, resistants might be expected to be more virulent, and actual observation seems to have confirmed the expectations in many instances (2-7). However, our experiments fail to support this notion since, at least in the case of *B. pestis caviæ*, resistants have been found repeatedly to be devoid of virulence. On the other hand, only cultures susceptible to lysis have been found to be pathogenic. In a measure, as cultures of resistants recover their susceptibility to lysis, they again become virulent. The cultures which fail to become susceptible to lysis, under the conditions of the experiment, remain avirulent. The fact that some resistant strains may not become susceptible, after having been carried free from bacteriophage for 200 successive transfers in broth, seems to indicate that the production of resistants is not a phenomenon of active "hereditary" immunization (12), but rather a result of irreversible variation, somewhat analogous to that observed in pneumococcus cultures (13, 14). It has been shown by Arkwright (15), Gratia (16), and others that cultures of bacteria of the colon-typhoid group can be normally dissociated into a number of variants of which at least two are manifestly different in the appearance of their colonies. Gratia (17) found later that the variants normally possess different resistance

no time was it possible to isolate the few susceptible individuals which must have appeared temporarily in such cultures, to be immediately overgrown by the resistants or to be destroyed by the phage. Since our first observations concerning the behavior of resistants obtained

Protocol VIII.

Attempt to Cause Reversion in the Presence of Antiphage Serum.

No. of transfers previous to test	Culture medium	Character of growth	Susceptibility to lysis as tested	
			In broth	On 1 per cent agar
1	Plain broth	Diffuse	—	—
	Broth + serum	Agglutinated	—	—
2	Plain broth	Diffuse	—	—
	Broth + serum	Agglutinated	—	—
4	Plain broth	Diffuse	—	—
	Broth + serum	Agglutinated	—	—
6	Plain broth	Diffuse	—	—
	Broth + serum	Agglutinated	—	—
7	Plain broth	Diffuse	—	—
	Broth + serum	Agglutinated	—	—
10	Plain broth	Diffuse	—	—
	Broth + serum	Agglutinated	—	—
17	Plain broth	Diffuse	—	—
	Broth + serum	Agglutinated	—	—
25	Plain broth	Diffuse	—	—
	Broth + serum	Sediment and diffuse overgrowth	—	—
32	Plain broth	Diffuse	—	—
	Broth + serum	Sediment and diffuse overgrowth	—	—

by means of bacteriophage W-178 (18), we have noted a similar failure of resistants to revert to susceptibility when other "strong" phages were used for their production. Although in every instance the resistants were tested and found not to carry phage, we attempted

Simultaneously with the recovery of susceptibility, the cultures of the first group regained a degree of virulence comparable to that of the parent culture of *B. pestis caviæ*. The cultures of the second group of resistants have failed thus far to recover virulence (10 months after isolation). The latter cultures, apart from lack of both virulence and susceptibility to lysis, are identical with the parent culture of *B. pestis caviæ*, as indicated by biochemical and antigenic properties.

Our findings offer evidence in favor of the view that resistant strains result from selection among variants already existing in the parent culture and do not arise through the inheritance of specific immunity properties produced by the action of phage.

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to lytic agents and may exhibit varying rates of growth (3). Thus, a bacterial culture may be considered in its cross-section (as indeed any other "population") as composed of individuals approaching a certain type, but occasionally lacking or taking on exaggerated characteristics. Depending upon the conditions of growth, some of the variants may find themselves favored by environment and may become quantitatively dominant. On the contrary, if the environment is changed so that it becomes incompatible with life or the multiplication of a certain type of variant, the latter is eliminated more or less completely, and the whole cross-section of the bacterial population in the culture changes accordingly, with more or less noticeable changes in the biologic activity of the culture as a whole. In the case of a highly specific, "weak" bacteriophage which displays activity only toward a comparatively narrowly defined type of individuals in the culture (as, for example, the phage W-Little), only the most susceptible individuals carrying the potential characteristic of virulence are destroyed, and if such a culture is allowed to grow in the absence of bacteriophage, the few remaining closely related individuals, which may carry the potential characteristic of virulence, begin to multiply anew and to produce the original cross-section of the culture. If, however, a phage of less specificity is used (as, for example, the "strong" phages W-178 or P.I.D.), it may happen that all the individuals carrying the characteristic of virulence are destroyed, and a permanently avirulent culture results.

SUMMARY AND CONCLUSIONS.

Resistants isolated from the overgrowth of cultures of *B. pestis cavize* (M. T. II) lysed by various strains of specific bacteriophage proved to be avirulent when administered to mice by feeding, or by intraperitoneal injection.

These cultures remained resistant to the action of bacteriophage so long as they were carried on agar. When transferred to broth, however, one group of resistants, namely, those isolated by means of "weak" phages, became susceptible to lysis after five to seven daily passages. The other group of resistants, isolated from the cultures lysed by one of the "strong" phages, failed to become susceptible to lysis even after nearly 200 passages in broth.

Culture Bovine 18 has not been used recently in infecting guinea pigs and consequently it is impossible to state how much of its virulence has been lost during its prolonged suspension in salt solution. Culture H 7156 has, however, recently been so used. In a group of 29 guinea pigs each received 0.1 mg. of H 7156 subcutaneously. The average length of life for the group was 164 days, with the extreme variations at 88 and 230 days. The animal receiving 0.1 mg. of the 325 day old suspension of H 7156 intraperitoneally died in 145 days. It would seem then that the virulence has not been greatly diminished.

TABLE I.

Culture	Age of suspension	Dose	Length of life, in days, following infection
	<i>days</i>	<i>mg.</i>	
18	310	0.1	93
18	310	1.0	160
18	330	0.1	98
18	330	1.0	128
7156	325	0.1	145
7156	325	1.0	108

SUMMARY.

Three suspensions of tubercle bacilli in physiological salt solution were still virulent for guinea pigs after 310, 325, and 330 days at refrigerator temperature. One of these cultures on which recent tests had been made had lost very little, if any, of its virulence for guinea pigs in 325 days.

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THE SURVIVAL OF THE TUBERCLE BACILLUS IN SUSPENSION IN PHYSIOLOGICAL SALT SOLUTION.

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The length of time that tubercle bacilli will live and maintain virulence when suspended in physiological salt solution has never been finally determined. The matter is of interest to those who use saline suspensions of the organisms in producing experimental infections. Webb, Ryder, and Gilbert (1) have noted that a saline suspension of tubercle bacilli was able to produce tuberculosis in guinea pigs after it had stood for as long as 87 days. They did not test its virulence for longer periods of time.

This note is to place on record the observation that three suspensions of tubercle bacilli in physiological saline were alive and virulent for guinea pigs after standing for periods of 310, 325, and 330 days, respectively, at refrigerator temperature.

The refrigerator used fluctuates about the freezing point, the fluctuations not being recorded. The suspensions were made by lightly grinding the moist bacilli from active glycerol agar cultures in an agate mortar, adding 0.85 per cent NaCl solution slowly at first and then more rapidly to the amount of 1 cc. per mg. of bacilli. The suspensions were stored in narrow neck, round flasks of pyrex glass stoppered with cotton. Evaporation was not measured but was slight in amount.

Two of these suspensions were of the same strain of organism, Bovine 18, and the other suspension was of a human type, 7156. Two guinea pigs were used in determining the viability of each suspension, one receiving 0.1 mg. of organisms intraperitoneally, and the second 1 mg. in like manner.

The results are presented in Table I.

All of these animals showed a well marked generalized tuberculosis and there was nothing atypical in the course of the disease or in the autopsy findings.

antigens were employed. Thus when horse serum or egg white was mixed with its respective antiserum and the precipitate removed by centrifugation, the supernatant liquid contained both antigen and antibody. With crystalline egg albumen the results were not the same. In these experiments he showed that both antigen and antibody never occurred together in the supernatant liquid. The fact that the presence of a third colloid failed to interfere with the interaction between antigen and antibody was demonstrated in his experiments.

The work of Opie³ follows more closely the phenomena subsequent to the therapeutic administration of horse serum. From his first series of experiments it is evident that after injection of horse serum into the blood stream of rabbits, precipitin for horse serum appears in the blood before the horse serum proteins entirely disappear. This he attributes to the composite antigenic nature of the horse serum, and suggests that certain of the contained proteins give rise to antibodies much more rapidly than certain others. When similar experiments were performed with a relatively pure crystallized egg albumen the findings were different. It is clear from Opie's findings that when crystallized egg albumen is employed as antigen the corresponding antibody and uncombined egg albumen do not appear in the blood together.

Ascoli⁴ had called attention to the fact that after the injection of egg white it appeared in the urine. Opie⁵ also found that crystallized egg albumen appeared in the urine subsequent to injection. Therefore, if such be the case, the injection of crystallized egg albumen hardly affords a parallel to the administration of foreign blood proteins. It is well known that the administration of large amounts of horse serum for therapeutic purposes does not result in the appearance of the foreign serum proteins in the urine provided the kidney is intact. The writer has on several occasions injected 5 or 6 cc. of cow serum into the peritoneal cavity of normal rabbits but in no instance has it been possible to detect it in the urine, although its presence in the rabbit's blood serum could readily be determined.

It seemed necessary to study the behavior of crystallized egg albumen after its injection to determine whether its rapid elimination might not afford an explanation for the fact that antibody failed to appear while the antigen was still present. The experiments indicate that the behavior of egg albumen is in certain respects different from that of foreign blood proteins, so that in the second series of observations casein, an antigen which on the whole affords an analogy to the blood proteins in its general behavior, was used.

³ Opie, E. L., *J. Immunol.*, 1923, viii, 55.

⁴ Ascoli, M., *Münch. med. Woch.*, 1902, xlix, 398.

⁵ Opie, E. L., *J. Exp. Med.*, 1924, xxxix, 659.

THE BEHAVIOR IN VIVO OF CERTAIN RELATIVELY PURE ANTIGENS.

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The fact that certain proteins, such as those of blood serum, when introduced into the body will persist in the circulation for considerable periods is well known. It is also true that in certain individuals precipitin will appear in the blood before the antigen has disappeared. Thus the paradox of antigen and antibody occurring in the blood at the same time is frequently encountered. Several explanations of this phenomenon have been offered. Some have favored the view that an excess of colloid will inhibit the combination of antibody and antigen. Others have pointed out that such substances as egg white and serum are composed of several proteins and that the precipitin which appears in the blood may really be due to a single antigen which has been exhausted during the production of the antibody. The resulting antibody would react with a portion of the complex antigen and the remaining proteins would still be capable of reacting with the complex precipitin.

During the last few years the view that the paradox is explicable on the basis of multiple antigens has come into considerable prominence although in the main relatively few experiments have been reported to support this view. Those of Bayne-Jones¹ cannot be said to do so. He was able to show that rabbits immunized with crystallized egg albumen, or edestin, until their serum contained precipitin in considerable concentration would on reinjection of the specific antigen contain both antigen and antibody for a period of 48 hours. He further brought out the fact that *in vitro* much the same phenomenon was observed when antigen and antibody were mixed and the resulting precipitin centrifuged out. The supernatant fluid contained both antibody and antigen. Previously, however, R. Weil's² experiment indicated that this phenomenon held true only when multiple

¹ Bayne-Jones, S., *J. Exp. Med.*, 1917, xxv, 837.

² Weil, R., *J. Immunol.*, 1916, i, 19.

Experiment 2.—Rabbit 2, weighing 2,405 gm., was injected intravenously, at 9.30 a.m. with 3 cc. of 5 per cent crystalline egg albumen. An hour later it was bled and at intervals throughout the day. Urine was passed 5½ hours after the first injection. At 4.45 p.m., 3 cc. of the albumen solution was injected into the ear vein. As in the previous experiment, the blood was regularly tested for egg albumen and its antibody. The urine was also tested for antigen. Inasmuch as the experiment added little to the development of precipitin, the details will not be given. It is sufficient to state that antibody appeared in the blood on the 7th day. The results of the tests of the blood serum and urine for egg albumen are recorded in Tables IV and V.

TABLE V.

Examination of the Urine of Rabbit 2 for Egg Albumen after Its Intravenous Injection.

Time urine was voided	Dilutions of urine tested							
	1:0	1:1	1:5	1:10	1:20	1:40	1:80	1:160
5½ hrs. after 1st injection.....	—	—	±	++	++	++	+	+
During night following 2nd injection (from 2 to 16 hrs. after 2nd injection).....	±	±	+	+	++	++	+	±
18 hrs. after 2nd injection.....	±	±	±	±	±	—	—	—
During 2nd night following 2nd injection.....	—	—	—	—	—	—	—	—
63 hrs. after 2nd injection.....	—	—	—	—	—	—	—	—
72 " " " "	—	—	—	—	—	—	—	—
4 days " " " "	—	—	—	—	—	—	—	—

The findings in the second experiment fully confirm those of the first. The contention that crystallized egg albumen is rapidly eliminated from the circulation is well borne out. It can be readily detected in the blood during the first 3 hours following its administration, but by the end of the 4th hour it has decreased considerably. After 7 hours only a trace remains and after 18 hours it can no longer be detected. The precipitin used in the observations would react with as little as 1/700,000 gm. of the antigen. The passage of the albumen through the kidney compares in a rough way with the decline in the circulation. In both instances the urine contained the antigen in considerable concentration as early as 6 hours after its injection. Egg albumen could be demonstrated only for a period of 1 or at most 2 days in the urine. The specific antibody appeared in the blood in one instance in 7 and in the other in 10 days after the injection of the antigen.

EXPERIMENTAL.

Rabbits, 2,500 gm. in weight, were immunized with various antigens and after sera of sufficient titer were obtained they were bled and the serum stored in the refrigerator. The specific precipitin was used to test the blood serum and urine for the presence of antigen. The test rabbits were of about the same age and size. The injections were made intravenous or intraperitoneal and the animals bled at frequent intervals from the ear vein. The blood was collected in sterile test-tubes and permitted to clot in a thin layer, in the incubator, and when sufficient serum had oozed out it was collected and centrifuged. In this way a clear serum was available for the tests within 2 or 3 hours.

The naturally voided urine was collected in clean bottles. It was filtered twice through filter paper.

TABLE I.

The Results of the Tests for the Presence of Antigen in the Blood Serum of Rabbit 1 Injected Intravenously with Crystallized Egg Albumen.

Time after last injection that sample was taken	Amount of serum tested				
	1.0 cc.	0.5 cc.	0.2 cc.	0.1 cc.	0.05 cc.
18 hrs.	—*	—	—	—	—
42 "	—	—	—	—	—
3 days	—	—	—	—	—
4 "	—	—	—	—	—

* Since crystallized egg albumen reacts powerfully with its antibody, the intensity of the reaction has been recorded as follows: + + +, a heavy precipitate; + +, a moderate precipitate; +, slight but definite precipitation; ±, turbidity with a trace of precipitate.

The crystallized egg albumen was prepared by precipitation with ammonium sulfate after the method of Hopkins and Pinkus.⁶ It was recrystallized four times. For the casein I am indebted to Dr. J. H. Northrop of The Rockefeller Institute. It was prepared by his method.⁷

Experiment 1.—Rabbit 1. Weight, 2,245 gm. On Nov. 2, 1925, at 9.45 a.m., received 3 cc. of a 5 per cent solution of crystallized egg albumen intravenously. The animal urinated at 4.10 p.m. At 4.45 p.m., 2.5 cc. of the egg albumen was again injected into the ear vein. A blood sample was taken on Nov. 3, at 10 a.m. Blood for the tests was drawn daily and the urine was collected during the next

⁶ Hopkins, F. G., and Pinkus, S. N., *J. Physiol.*, 1898–99, xxiii, 130.

⁷ Northrop, J. H., *J. Gen. Physiol.*, 1922–23, v, 749.

of normal rabbit serum a ring would form at the point of contact between the two liquids and after mixing precipitation would occur. Its use for this reason was discontinued.

Gay and Robertson⁸ and Wells and Osborne⁹ showed that purified casein is antigenic. By the repeated intraperitoneal injection of a 1 per cent solution of casein a precipitin was obtained which would in amounts of 0.1 or 0.2 cc. react with as little as 1/100,000 gm. of casein. As a rule the distinct line at the union of serum and casein occurred promptly. In the higher dilutions there is little actual precipitation but the mixed liquids promptly become turbid.

That casein, on the whole, meets the requirements of a relatively pure protein which will persist in the circulation for a considerable period is brought out in the following observations.

Experiment 3.—On different occasions rabbits were injected with casein. The casein was dissolved in N/20 NaOH and sterile salt solution. The solution was injected intraperitoneally in 3 doses during the day. Rabbit 3 received 0.6 gm. of casein; Rabbit 4 0.5 gm.

All the voided urine was collected and tested during the next 5 days. The blood serum from both rabbits was tested at frequent intervals for casein with the anticasein serum. As a control procedure tubes of the experimental animals' sera were incubated and refrigerated. In this way an accurate comparison of turbidity was possible. The urine was twice filtered through filter paper and tested in the same manner. Control tubes of the same sample were always incubated and refrigerated. The tests for casein precipitin were carried out in the usual manner. 0.2 cc. of the rabbit's serum was added to 1 cc. of the various dilutions of the antigen. The results are given in Table VI.

It is apparent that casein, like the blood proteins, remains in the circulation for considerable periods. Even as long as 12 and 13 days after its injection the serum responds weakly to the specific test. Like the serum proteins it fails to pass through the kidney in quantities sufficient to be detected. In both instances antibody was recognized in the blood serum while the casein was still in the circulation.

DISCUSSION AND SUMMARY.

The experiments are of interest in several respects. It is clear that crystallized egg albumen is rapidly eliminated from the circula-

⁸ Gay, F. P., and Robertson, T. B., *J. Exp. Med.*, 1912, xvi, 470.

⁹ Wells, H. G., and Osborne, T. B., *J. Infect. Dis.*, 1921, xxix, 200.

Since crystallized egg albumen fails to meet the general requirements, the number of relatively pure proteins of general availability is somewhat limited. Bayne-Jones used with considerable success

TABLE VI.

The Behavior of Casein and Its Antibody in Rabbits 3 and 4.

Rabbit No.	Days after injection	Test of serum for casein in		Test of urine for casein in			Test of serum for antibody			
		1.0 cc.	0.1 cc.	1.0 cc.	0.5 cc.	0.1 cc.	Dilutions of antigen			
							1:100	1:200	1:400	1:800
3	1	+	*	—	—	—	—	—	—	—
	2	+	—	—	—	—	—	—	—	—
	3	+	±	—	—	—	—	—	—	—
	4	+	±	—	—	—	—	—	—	—
	6	+	?	—	—	—	—	—	—	—
	7	+	?	—	—	—	—	±	±	—
	8	+	?	—	—	—	+	+	+	—
	9	+	—	—	—	—	+	+	+	—
	10	+	—	—	—	—	+	+	+	—
	11	±	—	—	—	—	±	+	+	+
	13	±	—	—	—	—	±	+	+	+
	14	±	—	—	—	—	±	+	+	+
	16	—	—	—	—	—	—	±	+	+
4	1	—	—	—	—	—	—	—	—	—
	2	—	—	—	—	—	—	—	—	—
	3	+	—	—	—	—	—	—	—	—
	4	+	—	—	—	—	—	—	—	—
	5	+	—	—	—	—	—	—	—	—
	7	+	—	—	—	—	—	—	—	—
	8	+	—	—	—	—	—	—	—	—
	9	+	—	—	—	—	±	±	±	—
	10	±	—	—	—	—	±	+	+	—
	11	±	—	—	—	—	+	+	+	—
	12	±	—	—	—	—	+	+	+	—
	14	—	—	—	—	—	+	+	+	—

* + indicates a well defined turbidity; ±, a slight turbidity.

the globulin edestin. In my hands edestin proved unsatisfactory. When dissolved in sufficiently strong alkali it could not be injected with safety. If by titration one attempted to reduce the free alkali it would give a clear solution, but on the addition of small amounts

this time there is constant union of antigen and antibody within the blood, with the slow utilization of the antigen and a slight utilization of the antibody which is made up by a slow increase from the body cells. Thus there would be a period in which considerable antigen would be present with weak antibody, succeeded by a second period when the amount of antigen would be small with well defined antibody, and finally only antibody. Certain observations tend to support such a view. Bayne-Jones injected rabbits whose serum contained precipitin from egg albumen with this substance and noted the occurrence of both antigen and antibody for a period of 48 hours. Some of his experiments *in vitro* are equally suggestive. In one instance a rabbit well immunized with egg albumen was injected intravenously with this substance. An hour later it was bled and the stored serum refrigerated for a period. During this time there was a slow spontaneous precipitation with a decline in both precipitin and antigen titer, but even after 6 days both were present. After a longer period only antigen remained. P. A. Lewis and D. Loomis¹¹ have shown that an injection of sheep red blood cells in guinea pigs results in a well defined hemolysin titer about the 9th day, followed by a definite decline, with a secondary rise in hemolysin until the peak is reached on the 20th day.

It becomes evident, then, that the reaction of the rabbit to a single injection of a relatively pure protein will depend on the character of the protein injected. When crystallized egg albumen is administered it is rapidly eliminated from the circulation. The rapid disappearance of the egg albumen from the blood stream is partly accounted for by its prompt elimination through the urine. Antibody appears in the serum from the 7th to the 10th day. Casein behaves differently. It persists in the blood for a considerable period; after the 7th or 8th day both antigen and antibody may be demonstrated in the blood. Casein cannot be detected in the urine following its injection into the body. The behavior of casein within the body affords an analogy with the conditions frequently noted after the administration of foreign serum, in both cases both antigen and antibody may be present in the circulation together.

¹¹ Lewis, P. A., and Loomis, D., *J. Exp. Med.*, 1924, xl, 503.

tion and in the experiments cited it could no longer be detected after 18 or 19 hours. A considerable portion of it rapidly passes through the kidney in an apparently unaltered state. Evidently this passage begins almost at once and may continue for a day or two. In an experiment not reported in this paper, egg albumen appeared in naturally voided urine 2 hours following its injection into the peritoneal cavity. In the experiments reported no urine was voided until $5\frac{1}{2}$ and $6\frac{1}{2}$ hours following intravenous administration, but in each instance egg albumen was present in considerable amounts. However, sufficient egg albumen must have been utilized to produce antibody. It is hardly to be expected that such a protein, whose elimination is so rapid, could persist unaltered within the body and reappear within the circulation coincident with its antibody. The behavior of the protein cannot be ascribed to alterations which may have taken place during the process of crystallization since Ascoli showed that the proteins of egg white readily pass from the circulation into the urine. Certain observations of the writer confirm this point. The experience of Alexander, Becke, and Holmes¹⁰ who exposed sensitized guinea pigs to sprays of dilute egg white with the result that 80 per cent of the animals developed symptoms of anaphylaxis, further strengthens the contention that certain of the membranes are readily permeable for the proteins of egg.

The conditions following the injection of casein are different. There is no appreciable passage through the kidney. Casein is present within the circulation for a considerable period; it could be detected in the blood serum 12 and 13 days after its introduction into the peritoneal cavity. Antibody appeared on the 7th and 8th days, respectively, so that both antigen and antibody were present in the serum for a period of 3 or 4 days. The phenomenon of antigen and antibody occurring together might be explained on the ground that certain proteins are utilized slowly and that the antibody found in the blood, usually after the 7th day, results from the portion of antigen first utilized. During the next few days a continual supply of antibody enters the circulation and during the period there is a steady utilization of the antigenic substance; it is possible that during

¹⁰ Alexander, H. L., Becke, W. G., and Holmes, J. A., *J. Immunol.*, 1926, xi, 175.

TABLE I.

Correlation Coefficients for Actual and Relative Organ Weight with Gross Body Weight.

	Actual	Relative
Net body weight.....	+0.958 \pm 0.002	
Hcart.....	+0.755 \pm 0.011	-0.169 \pm 0.026
Testicles.....	+0.555 \pm 0.019	+0.101 \pm 0.028
Kidneys.....	+0.471 \pm 0.021	-0.443 \pm 0.021
Gastrointestinal mass.....	+0.456 \pm 0.021	-0.311 \pm 0.024
Brain.....	+0.451 \pm 0.025	-0.761 \pm 0.013
Suprarenals.....	+0.364 \pm 0.023	-0.066 \pm 0.027
Hypophysis.....	+0.343 \pm 0.024	-0.402 \pm 0.022
Mesenteric lymph nodes.....	+0.307 \pm 0.03	-0.143 \pm 0.032
Liver.....	+0.291 \pm 0.024	-0.301 \pm 0.024
Thyroid.....	+0.248 \pm 0.025	-0.019 \pm 0.027
Deep cervical lymph nodes.....	+0.246 \pm 0.031	-0.058 \pm 0.033
Thymus.....	+0.222 \pm 0.025	-0.160 \pm 0.026
Parathyroids.....	+0.221 \pm 0.025	-0.174 \pm 0.026
Pineal.....	+0.193 \pm 0.026	-0.316 \pm 0.024
Popliteal lymph nodes.....	+0.190 \pm 0.028	-0.271 \pm 0.027
Spleen.....	+0.186 \pm 0.026	-0.113 \pm 0.026
Axillary lymph nodes.....	+0.074 \pm 0.029	-0.321 \pm 0.026

TABLE II.

Correlation Coefficients for Actual and Relative Organ Weight with Net Body Weight.

	Actual	Relative
Gross body weight.....	+0.937 \pm 0.003	
Heart.....	+0.739 \pm 0.012	-0.217 \pm 0.025
Testicles.....	+0.493 \pm 0.021	+0.018 \pm 0.028
Kidneys.....	+0.452 \pm 0.021	-0.473 \pm 0.021
Suprarenals.....	+0.415 \pm 0.022	+0.027 \pm 0.027
Brain.....	+0.392 \pm 0.027	-0.830 \pm 0.010
Hypophysis.....	+0.353 \pm 0.023	-0.449 \pm 0.021
Thyroid.....	+0.261 \pm 0.025	+0.013 \pm 0.027
Mesenteric lymph nodes.....	+0.251 \pm 0.031	-0.222 \pm 0.031
Gastrointestinal mass.....	+0.245 \pm 0.025	-0.448 \pm 0.021
Parathyroids.....	+0.242 \pm 0.025	-0.139 \pm 0.026
Deep cervical lymph nodes.....	+0.228 \pm 0.032	-0.103 \pm 0.033
Liver.....	+0.214 \pm 0.025	-0.417 \pm 0.022
Thymus.....	+0.213 \pm 0.025	-0.171 \pm 0.026
Pineal.....	+0.199 \pm 0.026	-0.323 \pm 0.024
Spleen.....	+0.143 \pm 0.025	-0.166 \pm 0.026
Popliteal lymph nodes.....	+0.116 \pm 0.029	-0.290 \pm 0.027
Axillary lymph nodes.....	+0.063 \pm 0.029	-0.336 \pm 0.026

THE RELATION BETWEEN BODY AND ORGAN WEIGHTS IN THE RABBIT.

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In the previous paper of this series (1), we summarized the results of that part of our study of organ weights the immediate object of which was to define conditions that obtain in normal stock rabbits with respect to mean weights, the tendency to the occurrence of variations in weight, and the probable limits of variation for different organs. As a further contribution to the general problem of physical constitution and as an approach to the study of conditions that determine or affect the weights of organs, we have undertaken an investigation of the normal relation that obtains between body and organ weight and between the weight of one organ and that of another. The purpose of this paper is to report the relation found between body and organ weight as indicated by correlation coefficients and a comparison of group means.

Methods and Material.

The results to be reported are based on data from 645 male rabbits of various breeds. The series contained a few young and a few old animals but the great majority of the animals were between 6 months and 2 years old with about an equal division between those that were sexually mature but not full grown and those that had attained sexual maturity and full growth. All of the animals were in apparent good health but on postmortem examination many of them showed active or healed lesions of some kind. In the present investigation no discrimination was made on the basis of age, breed, or the presence of lesions.

The actual weight and the weight of organs per kilo of net body weight¹ (relative

¹ Net body weight is the gross weight of the animal minus the weight of the gastrointestinal mass as defined in the first paper of this series (Brown, W. H., Pearce, L., and Van Allen, C. M., *J. Exp. Med.*, 1925, xlii, 69).

TABLE III—*Conclud.*

Gross body weight	No. of animals	Suprarenals		Hypophysis		Mesenteric lymph nodes		Liver		Thyroid		Deep cervical lymph nodes	
		Actual	Relative	Actual	Relative	Actual	Relative	Actual	Relative	Actual	Relative	Actual	Relative
kg.		gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.
1.400	1	0.228	0.194	0.030	0.0255			55.0	46.8	0.175	0.149		
1.563	2	0.505	0.3955	0.0255	0.0208			83.0	68.9	0.1625	0.1311		
1.633	6	0.275	0.2097	0.0237	0.0175	3.03	2.38	61.3	46.8	0.1725	0.1321	0.0988	0.0772
1.750	20	0.3222	0.2259	0.0264	0.0187	2.67	1.92	75.9	54.1	0.2864	0.2024	0.1114	0.0782
1.840	36	0.3285	0.2204	0.0259	0.0176	2.48	1.67	76.5	52.2	0.1866	0.1263	0.1252	0.084
1.933	56	0.3238	0.2083	0.0253	0.0164	3.18	2.05	80.1	51.7	0.1897	0.1216	0.1414	0.0914
2.033	89	0.3239	0.1984	0.0269	0.0166	3.34	2.06	82.3	50.6	0.1944	0.1194	0.1558	0.0959
2.134	83	0.3339	0.1944	0.0273	0.0159	3.26	1.91	82.0	48.0	0.217	0.1266	0.1458	0.0858
2.235	78	0.3694	0.2038	0.0272	0.0150	3.70	2.04	83.4	47.0	0.2079	0.1149	0.1428	0.0785
2.338	75	0.4226	0.2203	0.0282	0.0148	3.38	1.79	92.5	47.4	0.2269	0.1186	0.1484	0.0787
2.436	59	0.4332	0.2164	0.0307	0.0154	3.55	1.79	89.6	44.9	0.2468	0.1221	0.1629	0.0809
2.531	44	0.4358	0.2104	0.0301	0.0142	3.61	1.75	91.0	44.1	0.3095	0.1467	0.1758	0.0851
2.638	23	0.4239	0.1964	0.0311	0.0144	3.95	1.84	84.0	43.7	0.2507	0.1154	0.1678	0.0781
2.732	25	0.4436	0.1947	0.0301	0.0133	4.09	1.79	91.2	40.2	0.2835	0.1239	0.2024	0.0884
2.815	13	0.4117	0.1801	0.0314	0.0138	3.82	1.68	96.9	42.8	0.3202	0.1394	0.2511	0.1108
2.936	11	0.5868	0.2405	0.0319	0.0130	4.19	1.69	92.0	37.8	0.3905	0.1587	0.1969	0.0796
3.047	9	0.5177	0.2006	0.0315	0.0123	4.03	1.57	93.8	36.8	0.2611	0.1019	0.1979	0.0777
3.107	7	0.7000	0.2996	0.0343	0.0142	4.65	1.80	101.6	39.1	0.355	0.1509	0.1238	0.0598
3.200	2	0.475	0.1877	0.0365	0.0144	8.10	3.23	94.5	37.4	0.2075	0.0821	0.185	0.0739
3.350	4	0.436	0.1517	0.0302	0.0106	3.83	1.36	114.8	40.6	0.3775	0.1343	0.2083	0.0753
3.450	1	0.635	0.217	0.032	0.0109	4.00	1.37	120.0	41.0	0.436	0.149	0.225	0.0769
3.500	1	0.590	0.1967	0.030	0.0100	4.85	1.61	90.0	29.9	0.235	0.0784	0.125	0.0416
Correlation coefficient		+0.364 ±0.023	-0.066 ±0.027	+0.343 ±0.024	-0.402 ±0.022	+0.307 ±0.03	-0.143 ±0.032	+0.291 ±0.024	-0.301 ±0.024	+0.248 ±0.025	-0.019 ±0.027	+0.246 ±0.031	-0.058 ±0.033

TABLE III.
Group Means for Body (Gross) and Organ Weights.

Gross body weight	No. of animals	Net body weight		Heart		Testicles		Kidneys		Gastrointestinal mass		Brain	
		gm.	Relative gm.	Actual gm.	Relative gm.	Actual gm.	Relative gm.	Actual gm.	Relative gm.	Actual gm.	Relative gm.	Actual gm.	Relative gm.
kg.													
1.400	1	1175	3.36	3.95	2.74	3.22	17.28	20.29	191.5	225.0	8.16	9.38	8.16
1.563	2	1245	2.91	3.53	3.06	3.74	8.87	11.04	261.7	317.5	6.32	8.18	6.32
1.633	6	1306	2.74	3.57	2.27	1.74	7.97	10.44	252.9	327.5	6.04	8.44	6.04
1.750	20	1415	2.89	4.08	2.20	3.09	7.90	11.17	245.9	335.7	5.80	8.60	5.80
1.840	36	1472	4.27	2.90	2.53	3.80	7.99	11.76	250.6	365.1	5.69	8.87	5.69
1.933	56	1553	4.62	2.98	2.41	3.74	7.73	11.98	244.4	380.0	5.46	8.86	5.46
2.033	89	1629	2.99	4.86	2.53	4.15	7.52	12.20	248.7	401.6	5.39	9.23	5.39
2.134	83	1717	2.93	5.02	2.45	4.16	7.26	12.46	243.6	415.2	5.12	9.28	5.12
2.235	78	1811	2.81	5.11	2.61	4.72	7.04	12.71	233.9	420.0	4.87	9.26	4.87
2.338	75	1908	2.82	5.37	2.60	5.02	6.84	13.01	225.7	439.0	4.63	9.27	4.63
2.436	59	2005	2.87	5.73	2.48	4.99	6.88	13.77	217.2	431.1	4.54	9.43	4.54
2.531	44	2070	2.76	5.71	2.70	5.58	6.62	13.68	222.2	457.3	4.29	9.24	4.29
2.638	23	2160	2.74	5.94	2.40	5.29	6.32	13.67	220.6	474.3	4.27	9.76	4.27
2.732	25	2272	2.71	6.42	2.58	5.87	6.27	14.28	197.1	445.4	4.30	9.82	4.30
2.815	13	2282	2.79	6.35	2.73	6.19	6.33	14.43	233.0	527.3	3.91	9.65	3.91
2.936	11	2440	2.82	6.85	2.66	6.52	6.11	16.44	200.3	485.0	3.95	10.06	3.95
3.047	9	2562	2.75	7.03	2.52	6.43	6.65	15.53	187.5	485.2	4.09	9.39	4.09
3.107	7	2605	2.97	7.12	2.69	6.33	6.65	15.81	191.9	497.0	4.51	11.30	4.51
3.200	2	2530	2.94	7.45	2.69	7.30	5.82	16.20	166.3	670.0	3.66	10.28	3.66
3.350	4	2855	2.72	7.74	2.93	8.58	5.16	16.48	179.5	470.0	3.77	11.03	3.77
3.450	1	2925	2.46	7.20	2.98	8.58	5.23	15.10	162.8	525.0			
3.500	1	3010	2.55	7.68	2.98	8.98	5.23	15.78		490.0			
Correlation coefficient		+0.958 ±0.002	-0.169 ±0.026	+0.755 ±0.011	+0.101 ±0.028	+0.555 ±0.019	-0.471 ±0.021	+0.443 ±0.021	+0.456 ±0.021	+0.451 ±0.025	-0.761 ±0.013		

TABLE IV.
Group Means for Body (Net) and Organ Weights.

Net body weight	No. of animals	Gross body weight	Heart		Testicles		Kidneys		Suprarenals		Brain	
			Actual	Relative	Actual	Relative	Actual	Relative	Actual	Relative	Actual	Relative
kg.		gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.
1.163	2	1475	3.89	3.34	3.82	3.30	15.26	13.09	0.264	0.228	9.38	8.16
1.247	4	1681	3.93	3.15	2.94	2.37	10.20	7.91	0.288	0.223	8.34	6.69
1.358	14	1733	3.81	2.80	2.62	1.92	11.14	8.19	0.326	0.240	8.53	6.26
1.443	38	1827	4.33	3.0	4.89	3.34	11.42	8.17	0.308	0.211	8.72	6.06
1.550	87	1964	4.66	3.0	3.92	2.52	12.09	7.81	0.313	0.202	8.85	5.70
1.650	90	2067	4.87	2.95	4.21	2.52	12.62	7.66	0.328	0.200	9.07	5.50
1.745	79	2151	4.98	2.85	4.09	2.34	12.34	6.93	0.349	0.200	8.96	5.13
1.844	87	2260	5.24	2.83	4.96	2.69	13.05	7.07	0.372	0.202	9.46	5.11
1.952	70	2382	5.46	2.79	5.14	2.64	13.02	6.69	0.451	0.232	9.18	4.70
2.046	59	2470	5.72	2.80	5.07	2.45	13.51	6.60	0.434	0.212	9.24	4.53
2.146	44	2596	5.86	2.73	5.48	2.54	13.79	6.42	0.427	0.197	9.41	4.38
2.243	23	2729	6.41	2.85	5.78	2.60	14.10	6.47	0.414	0.185	9.58	4.28
2.350	13	2796	6.86	2.93	6.48	2.76	14.66	6.25	0.559	0.238	9.66	4.10
2.461	12	2945	6.97	2.83	6.72	2.73	16.12	6.53	0.448	0.182	9.95	4.04
2.544	9	3044	6.51	2.77	6.34	2.77	15.55	6.56	0.583	0.254	10.01	4.45
2.655	9	3110	7.11	2.68	6.59	2.48	16.13	6.11	0.681	0.257	9.65	3.62
2.870	1	3300	7.34	2.56	9.12	3.15	18.13	6.31	0.475	0.166	9.70	3.38
2.942	3	3392	7.47	2.54	7.56	2.74	14.30	4.85	0.540	0.183	10.62	3.62
3.010	1	3500	7.68	2.55	8.98	2.98	15.78	5.23	0.590	0.197		
Correlation coefficient		+0.937	+0.739	-0.217	+0.493	+0.018	+0.452	-0.473	+0.415	+0.027	+0.392	-0.830
		±0.003	±0.012	±0.025	±0.021	±0.028	±0.021	±0.021	±0.022	±0.027	±0.027	+0.010

Gross body weight	No. of animals	Thymus		Parathyroids		Pituitary gland		Popliteal lymph nodes		Spleen		Axillary lymph nodes	
		Actual	Relative	Actual	Relative	Actual	Relative	Actual	Relative	Actual	Relative	Actual	Relative
gms.		gms.	gms.	gms.	gms.	gms.	gms.	gms.	gms.	gms.	gms.	gms.	gms.
1.400	1	1.280	1.090	0.010	0.00851	0.015	0.01276	0.180	0.1532	1.15	0.978	0.150	0.1276
1.563	2	2.103	1.720	0.015	0.01213	0.0135	0.011	0.230	0.1715	1.165	0.915	0.130	0.097
1.633	6	1.553	1.197	0.0115	0.00887	0.0163	0.01253	0.184	0.1421	0.787	0.603	0.115	0.0891
1.750	20	1.889	1.334	0.0136	0.00952	0.0144	0.01016	0.208	0.1473	0.753	0.528	0.1497	0.1063
1.840	36	1.991	1.291	0.01161	0.00791	0.0153	0.01041	0.232	0.1546	0.925	0.629	0.1667	0.1138
1.933	56	2.110	1.356	0.01141	0.00735	0.0148	0.0095	0.243	0.1565	0.943	0.602	0.1592	0.1019
2.033	89	2.234	1.375	0.01208	0.00744	0.0152	0.00931	0.252	0.1552	0.954	0.590	0.1687	0.1034
2.134	83	2.269	1.334	0.01197	0.00699	0.0155	0.00906	0.256	0.1492	1.042	0.609	0.1646	0.09597
2.235	78	2.240	1.236	0.0119	0.00661	0.0151	0.00828	0.261	0.1435	0.930	0.519	0.1824	0.1034
2.338	75	2.317	1.224	0.01343	0.00705	0.0158	0.00828	0.261	0.1377	1.044	0.550	0.1739	0.0915
2.436	59	2.356	1.175	0.01338	0.00671	0.0159	0.00793	0.263	0.1312	1.061	0.533	0.1805	0.0896
2.531	44	2.527	1.221	0.01495	0.00726	0.0179	0.00871	0.258	0.1254	1.170	0.572	0.1714	0.0832
2.638	23	2.740	1.272	0.01308	0.00607	0.0161	0.00749	0.285	0.1319	1.013	0.469	0.1794	0.0831
2.732	25	2.657	1.172	0.01356	0.00598	0.0163	0.00716	0.250	0.1100	1.181	0.522	0.164	0.0721
2.815	13	2.689	1.181	0.01346	0.00593	0.0155	0.00683	0.272	0.1200	1.249	0.552	0.1646	0.0734
2.936	11	2.599	1.066	0.01454	0.00593	0.0155	0.00632	0.256	0.1039	1.195	0.489	0.1511	0.0614
3.047	9	2.626	1.026	0.01511	0.00592	0.0197	0.00771	0.304	0.1198	1.216	0.487	0.1661	0.0653
3.107	7	2.739	1.054	0.02143	0.00917	0.021	0.00809	0.279	0.1052	1.502	0.574	0.173	0.0664
3.200	2	2.350	0.928	0.017	0.00671	0.022	0.00871	0.250	0.0999	1.895	0.749	0.175	0.0695
3.350	4	2.530	1.094	0.01625	0.00564	0.0205	0.00716	0.338	0.1195	0.995	0.350	0.2217	0.0776
3.450	1	3.100	1.060	0.012	0.0041	0.018	0.00616	0.320	0.1094	1.300	0.445	0.215	0.0735
3.500	1	1.890	0.630	0.032	0.01064	0.025	0.00834	0.470	0.1561	0.930	0.309	0.180	0.0598
Correlation coefficient		+0.222 ±0.025	-0.160 ±0.026	+0.221 ±0.025	-0.174 ±0.026	+0.193 ±0.026	-0.316 ±0.024	+0.190 ±0.028	-0.271 ±0.027	+0.186 ±0.026	-0.113 ±0.026	+0.074 ±0.029	-0.321 ±0.026

TABLE IV—Concluded.

Net body weight	No. of animals	Deep cervical lymph nodes		Liver		Thymus		Pineal gland		Spleen	
		Actual	Relative	Actual	Relative	Actual	Relative	Actual	Relative	Actual	Relative
		gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.
1.163	2			83.5	72.1	1.867	1.613	0.0150	0.0129	0.940	0.806
1.247	4	0.0938	0.0752	74.8	59.5	1.345	1.073	0.0155	0.0124	0.753	0.601
1.358	14	0.1008	0.0745	72.2	53.1	1.744	1.285	0.0138	0.0102	0.848	0.640
1.443	38	0.1285	0.0891	81.2	56.3	2.014	1.392	0.0144	0.0100	0.886	0.613
1.550	87	0.1392	0.090	82.4	53.1	2.024	1.310	0.0144	0.0093	0.955	0.620
1.650	90	0.164	0.0996	83.2	50.3	2.268	1.381	0.0158	0.0096	1.021	0.616
1.745	79	0.1447	0.0833	80.8	46.3	2.245	1.291	0.0159	0.0092	1.022	0.588
1.844	87	0.1437	0.0779	88.2	47.6	2.279	1.242	0.0153	0.0083	0.963	0.525
1.952	70	0.152	0.0777	87.3	44.7	2.334	1.196	0.0161	0.0082	1.053	0.539
2.046	59	0.161	0.0787	89.5	43.8	2.587	1.267	0.0166	0.0081	1.100	0.535
2.146	44	0.1754	0.0817	91.2	42.5	2.526	1.175	0.0164	0.0077	1.140	0.530
2.243	23	0.2196	0.0981	96.0	42.6	2.816	1.255	0.0153	0.0069	1.030	0.458
2.350	13	0.1781	0.0756	86.9	36.9	2.514	1.070	0.0166	0.0071	1.078	0.458
2.461	12	0.2205	0.0895	90.2	36.6	2.648	1.077	0.0188	0.0077	1.058	0.423
2.544	9	0.166	0.0656	90.5	35.6	2.285	0.897	0.0189	0.0074	1.334	0.525
2.655	9	0.1933	0.0726	96.3	36.3	2.282	1.153	0.0184	0.0069	1.551	0.586
2.870	1	0.150	0.0527	115.0	40.1	2.050	0.714	0.0200	0.0070	0.900	0.313
2.942	3	0.1875	0.0639	106.7	36.3	2.763	0.940	0.0210	0.0071	1.093	0.372
3.010	1	0.125	0.0416	90.0	29.9	1.890	0.630	0.0250	0.0083	0.930	0.309
Correlation coefficient		+0.228 ±0.032	-0.103 ±0.033	+0.214 ±0.025	-0.417 ±0.022	+0.213 ±0.025	-0.171 ±0.026	+0.199 ±0.026	-0.323 ±0.024	+0.143 ±0.025	-0.166 ±0.026

Net body weight	No. of animals	Hypophysis		Thyroid		Mesenteric lymph nodes		Gastrointestinal mass		Parathyroids	
		Actual	Relative	Actual	Relative	Actual	Relative	Actual	Relative	Actual	Relative
kg.		gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.
1.163	2	0.029	0.02492	0.1675	0.144	3.42	2.73	312.0	269.8	0.0125	0.01073
1.247	4	0.0245	0.01858	0.1513	0.1217	3.42	2.73	434.0	348.9	0.0113	0.009
1.358	14	0.0251	0.01849	0.1712	0.1264	2.38	1.74	375.0	276.1	0.0104	0.0077
1.443	38	0.0258	0.01791	0.2343	0.1632	2.97	2.06	381.0	267.2	0.0128	0.0089
1.550	87	0.0251	0.01682	0.185	0.1195	3.20	2.07	412.0	264.8	0.0112	0.0072
1.650	90	0.0274	0.01662	0.2101	0.1273	3.33	2.02	413.0	250.5	0.0129	0.0079
1.745	79	0.0269	0.01544	0.2089	0.1199	3.39	1.94	405.0	232.3	0.0118	0.0068
1.844	87	0.0278	0.01511	0.2096	0.1139	3.53	1.91	414.0	225.1	0.0122	0.0066
1.952	70	0.0286	0.01465	0.2262	0.1151	3.53	1.81	425.0	217.7	0.0137	0.0071
2.046	59	0.0306	0.01496	0.2688	0.131	3.31	1.62	423.0	206.8	0.014	0.0069
2.146	44	0.0303	0.01418	0.3021	0.1406	3.60	1.68	448.0	208.8	0.0139	0.0065
2.243	23	0.0291	0.01299	0.2723	0.1213	4.33	1.93	475.0	211.8	0.012	0.0054
2.350	13	0.031	0.0133	0.2943	0.1679	4.18	1.69	432.0	184.0	0.0125	0.0053
2.461	12	0.0324	0.01318	0.3977	0.1332	4.24	1.72	478.0	192.2	0.0165	0.0067
2.544	9	0.0335	0.0139	0.3733	0.1579	4.42	1.74	504.0	198.2	0.0172	0.0075
2.655	9	0.033	0.01244	0.3272	0.1232	4.14	1.56	448.0	168.7	0.0179	0.0064
2.870	1	0.035	0.0122	0.490	0.1716	2.72	0.95	430.0	149.8	0.016	0.0055
2.942	3	0.030	0.0102	0.3137	0.1067	4.60	1.56	450.0	153.0	0.0166	0.0057
3.010	1	0.030	0.00997	0.235	0.0784	4.85	1.61	490.0	162.8	0.032	0.0106
Correlation coefficient		+0.353	-0.449	+0.261	+0.013	+0.251	-0.222	+0.215	-0.448	+0.242	-0.139
		±0.023	±0.021	±0.025	±0.027	±0.031	±0.031	±0.025	±0.021	±0.025	±0.026

weight) were correlated with both gross and net body weight. The correlation coefficients are recorded in Tables I and II in the order of the magnitude of the coefficients obtained for actual weight. Space does not permit the publication of complete correlation tables but a summary of the group means is given in Tables III and IV arranged on the basis of increasing gross and net body weights respectively. The results for gross body weight are plotted in Text-fig. 1; those for net body weight do not differ sufficiently to warrant reproduction. In order to facilitate direct comparison the values for organ weight are plotted on the basis of a percentage deviation from the mean weight of the organ concerned for a given increase in body weight so that the scales of all curves are comparable. The significant parts of the curves are included between heavy perpendicular lines. The groups to the right or left of these lines contained only a few animals but the values are given as they were used in calculating the coefficients.

RESULTS.

The relations found between body and organ weights are presented in Tables I to IV and Text-fig. 1.

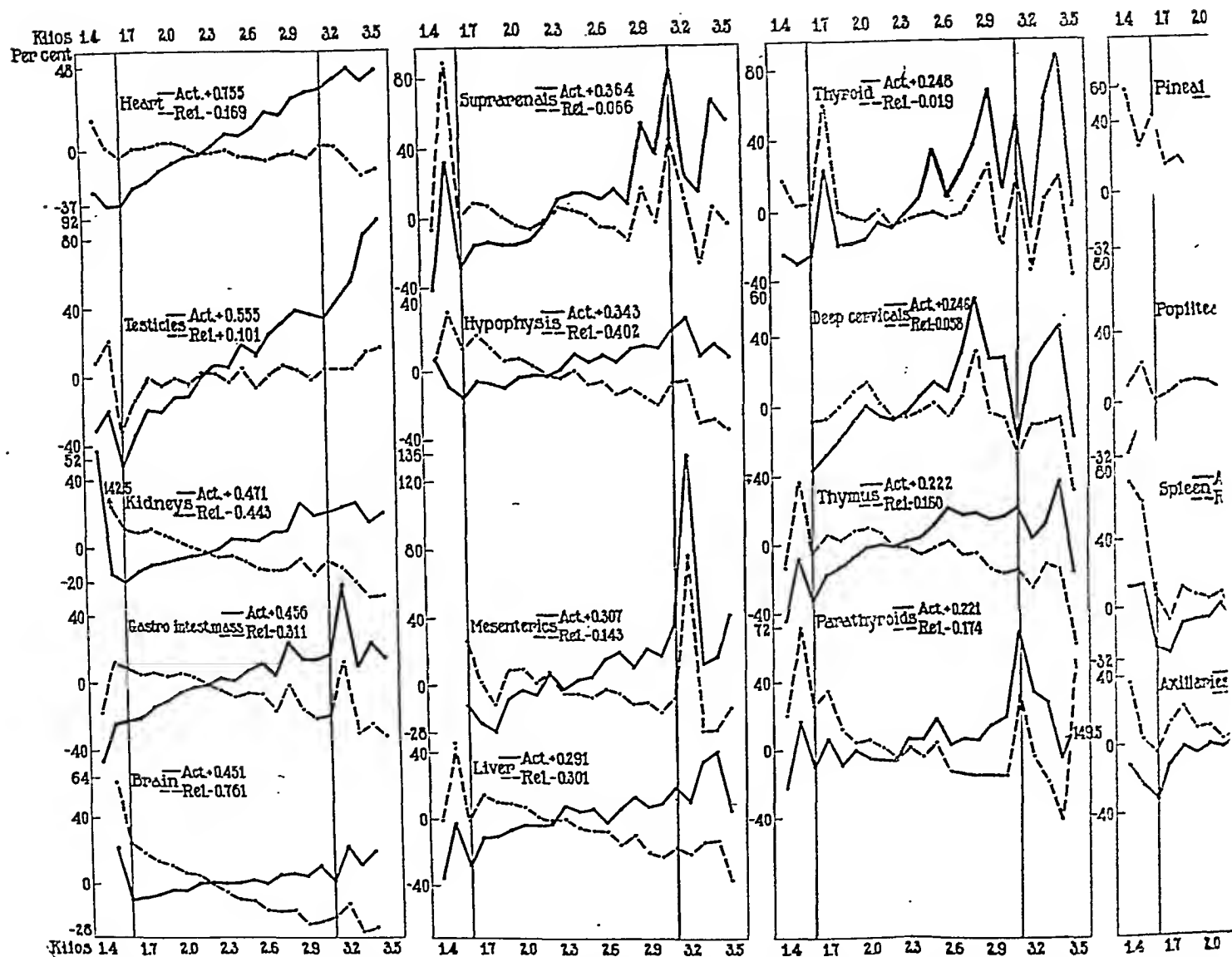
DISCUSSION AND CONCLUSIONS.

The results presented in Tables I to IV and Text-fig. 1 bring out a number of important points concerning the physical constitution of mature and apparently healthy stock rabbits. With very few exceptions, the ratio of the correlation coefficients to their probable errors (Tables I and II) is sufficiently large to warrant acceptance of the results as valid measures of the relation existing between body and organ weight, irrespective of the magnitude of the coefficients.

The coefficients show a great diversity of relations. In the first place, it will be seen that there is a *positive* relation of some kind between the actual weight of all organs and the body weight of the animal while the coefficients for relative weight are either *negative* or *approach a zero order*. That is to say, there is evidence of a common tendency on the part of all organs to weigh more or less according to the weight of the animal but, in only a few instances, is the difference in weight of such an order as to maintain a constant relation between body and organ weight; the weight of the organ per kilo of body weight varies and, as a rule, diminishes as the weight of the animal increases.

In estimating the degree of the correlation there are two values to

Net body weight kg.	No. of animals	Popliteal lymph nodes		Axillary lymph nodes	
		Actual gm.	Relative gm.	Actual gm.	Relative gm.
1.163	2	0.180	0.1532	0.150	0.1276
1.247	4	0.1725	0.1377	0.1388	0.1109
1.358	14	0.2418	0.1769	0.1527	0.1118
1.443	38	0.2311	0.1569	0.1607	0.1111
1.550	87	0.239	0.1551	0.1568	0.101
1.650	90	0.2546	0.1543	0.1705	0.1033
1.745	79	0.2533	0.1483	0.1659	0.0951
1.844	87	0.2639	0.143	0.1813	0.0984
1.952	70	0.2577	0.1319	0.1756	0.0897
2.046	59	0.2738	0.134	0.1845	0.090
2.146	44	0.2439	0.1135	0.1563	0.0728
2.243	23	0.2264	0.1232	0.1733	0.0773
2.350	13	0.2512	0.1068	0.160	0.0679
2.461	12	0.2845	0.1154	0.1702	0.0694
2.544	9	0.2225	0.0878	0.1292	0.0511
2.655	9	0.2681	0.0997	0.1584	0.0595
2.870	1	0.350	0.122	0.180	0.0627
2.942	3	0.3475	0.1182	0.2775	0.0945
3.010	1	0.470	0.1561	0.180	0.0598
Correlation coefficient		+0.116 ±0.029	-0.290 ±0.027	+0.063 ±0.029	-0.336 ±0.026



TEXT-FIG. 1. Relation of actual and relative organ weights to body weight plotted as a percentage deviation of the group means for organ values for all animals. (Act. = actual organ weight, Rel. = relative organ weight.)

plotting the group means for actual and for relative organ weight against the corresponding values for gross body weight (Table III). These curves show three distinct forms of relation: first, a linear increase in actual weight which is directly proportional to body weight; second, a straight line increase in actual weight which is of such an order as to produce a linear decrease in the weight of the organ per kilo of body weight; third, an increase in the actual weight of the organ over the lower ranges of body weight with the maintenance of a constant or diminishing level of actual weight over the middle and upper ranges which produces first an increase and then a decrease in the weight of the organ per kilo of body weight (popliteal and axillary lymph nodes).

Other curves appear to be modifications or combinations of these with the possible exception of the curve for the suprarenals. There is some indication that the change in the weight of the suprarenals is not of the order of a uniform progression but shows a diphasic condition characterized by stabilization of actual weights at successive levels or by a succession of increases and decreases in relative weight. It may be that this condition is referable to some factor other than body weight which, in the case of the suprarenals, is sufficiently potent to obscure the influence of the body weight factor.

Analyzing the results obtained from the standpoint of the correlation coefficients and the form of the relation shown, it will be seen that there are comparatively few organs the weights of which are closely related to body weight. The heart shows the closest correlation from every point of view. The coefficient for actual weight is much larger than that of any other organ while the coefficient for relative weight is comparatively small. This, in itself, suggests that there is a constant ratio between the weight of the heart and the weight of the body which holds for practically all ranges of weight within the limits of these observations. This conclusion is borne out by plotting the mean values for actual and relative weights (Table III) against body weight. The curve obtained (Text-fig. 1) shows a linear increase in the actual weight of the heart which is of such an order as to maintain the weight per kilo at a practically constant level. This may be taken as an example of almost perfect physical correlation between the weight of an organ which performs a mechanical function and that of the body which it serves.

be considered, first, the direct correlation between the actual weight of the organ and that of the body and, second, the inverse relation between the weight of the organ per kilo of body weight and the weight of the body. By reference to Tables I and II, it will be seen that there is no constant relation between these two values. The ideal condition of a high positive and a low negative coefficient, or a coefficient of the zero order, is shown by very few organs. In some cases this relation is reversed while in others the two sets of coefficients are of a comparable magnitude. If, however, we arrange the organs according to the magnitude of the correlation coefficients for actual weight with gross body weight, as in Table I, it will be seen that in general the so called major organs of the body show the highest values and the lymphoid organs the lowest with the endocrine glands occupying an intermediate position.

The situation presented by correlating relative organ weight with either gross or net body weight is entirely different. The coefficient obtained in this way serves as an inverse measure of the extent to which the increase in the actual weight of different organs approximates the ideal condition of the maintenance of a constant ratio between body and organ weight. In most instances the magnitude of the coefficient for relative weight is smaller than that for actual weight.

Similar conditions obtain when the weights of organs are correlated with net instead of gross body weight (Table II). The chief difference between the two sets of results is in the magnitude of the correlation coefficients for different classes of organs. By correlating actual organ weight with net body weight, the coefficients for the endocrine glands are increased while those for all other organs are either diminished or unaffected; in the case of relative weights, all values are increased with the exception of those for the thyroid, parathyroids, suprarenals, and testicles. While in most instances the change in the magnitude of the coefficient is comparatively small, the effect of this method of correlation is to strengthen the direct correlation of the endocrine glands and to weaken that of other organs.

A clearer conception of the form as well as the measure of the relation between body and organ weight may be gained by an examination of the curves in Text-fig. 1 which give the results obtained by

a slight and very uniform increase in the actual weight of the brain for animals weighing between 1500 and 2000 gm.; from 2000 to 2500 gm. it is doubtful whether there is any change but in still heavier animals there is apparently a second increase of the same order but less uniform than the first.

The organs considered above form a small group with comparatively close and clearly defined relations to body weight. At the opposite end of the scale, we find such purely lymphoid organs as the popliteal and axillary lymph nodes with negative coefficients that are distinctly larger than the positive coefficients for actual weight. The relation shown by these organs is of a comparatively low order and even this may be an effect of age rather than weight.

It will be seen that the mesenteric and deep cervical lymph nodes differ from the popliteal and axillary nodes; the coefficients for actual weight are larger and the negative coefficients are smaller so that the relation of these two masses of lymphoid tissue to body weight is not only closer than that of the superficial lymph nodes but is also of a different character (Text-fig. 1).

The conditions shown by the spleen and thymus are of especial interest. Their weights appear to be only slightly affected by body weight; the coefficients for actual weight are comparatively small but are slightly larger than those for relative weight so that the results agree with the superficial lymph nodes in one respect and with the deep lymph nodes in another. The closest analogy, however, is with the parathyroids which have coefficients that are almost identical with those of the thymus. This is of interest as we have additional evidence of a relation between these organs.

The endocrine glands show a variety of conditions. The suprarenals and the thyroid give results which differ chiefly with respect to the magnitude of the coefficients. The coefficients for the actual weight of the suprarenals are comparatively large while those for relative weight are very small giving a value for the direct relation which is considerably higher than that of any other organ in this group. The significant feature of the relation shown by these two organs is, however, the constancy of the weight per kilo of body weight within certain limits (Text-fig. 1).

The hypophysis and pineal gland show a relation to body weight

The testicles show a similar relation to body weight, giving a high correlation coefficient for actual weight and a small positive coefficient for relative weight, while the mean values (Table III) form a curve which shows the same relation between actual and relative weights on the one hand, and the weight of the body on the other, as the curve for the heart. This result may be regarded as highly significant and indicates that the generally recognized relation between testicular development and growth is of a very high order.

Among the organs studied, the testicles and the heart are the only ones that show a high correlation of this type. The kidneys, the gastrointestinal mass, and the brain come next in the order of magnitude of the correlation coefficients between actual and gross body weight. But, these organs show a negative coefficient for relative weight of a high order which would lead one to infer that the rate of increase in the actual weight of the organ is not proportional to the increase in body weight. By plotting the mean values (Table III, Text-fig. 1), we find that, within the limits of these observations, the increase in the weight of the kidneys is fairly uniform but the rate of increase is such that the weight per kilo of body weight diminishes at a rate which is approximately equal to that of the increase in actual weight, giving correlation coefficients of essentially the same magnitude ($+0.471 \pm 0.021$ and -0.443 ± 0.021). The gastrointestinal mass behaves in much the same manner but the coefficient for relative weight is smaller ($+0.456 \pm 0.021$ and -0.311 ± 0.024), and the regression in the weight of the mass per kilo of body weight is correspondingly less.

The coefficients for the liver ($+0.291 \pm 0.024$; -0.301 ± 0.024) are of a lower order than those for the kidneys and gastrointestinal mass but they show a similar relationship to body weight with a suggestion of a tendency toward an accentuation of the inverse relation which is brought out so strikingly in the case of the brain. The condition presented by the brain is the reverse of that shown by the organs mentioned above. The coefficient for actual weight is $+0.451 \pm 0.025$ while that for relative weight is -0.761 ± 0.013 . These values would lead one to expect a very uniform but very small increase in the actual weight of the brain with increasing body weight. The curve formed by the group means (Table III; Text-fig. 1) shows

This arrangement of organs takes into account structural and functional relationships as well as the correlation between body and organ weight. The organs studied are divided into three main groups; each of these groups contains three subdivisions, which differ with respect to the relative magnitude of the coefficients for actual and relative weights, arranged in the order of a diminishing direct or increasing inverse relation. The table may be read in any direction. In general, it will be seen that the magnitude of the coefficients for corresponding subdivisions, and hence the closeness of the relation between body and organ weight, diminishes from left to right; in like manner, the direct relation, which is strongest in the first subdivision of each group, diminishes and then changes to an inverse relation. The extreme conditions are represented by the upper left and the lower right divisions.

Tabulation of the results on the basis of the coefficients for net body weight, as has been pointed out above, merely accentuates the direct relation of the endocrine glands and the inverse relation of other organs with only a few minor changes in the actual arrangement of the organs in any given subdivision.

A number of interesting deductions may be drawn from this study. As has already been pointed out, the relations between body and organ weight are diverse. There are a number of organs that show a comparatively high and undoubtedly significant correlation with body weight but only a few that show a correlation of a very high order. In some cases it is the direct relation that is significant, in others, the indirect relation overshadows the direct and, in still other instances, the two are of about equal rank. There are only two clearly defined instances, however, of a direct relation between the actual weight of an organ and that of the body which is sufficiently close to maintain a constant ratio between body and organ weight. In most cases, the relations are such as to favor a diminishing weight per kilo of body weight. For animals weighing less than 2200 to 2300 gm., the weight of the organ exceeds the mean value for animals of all groups while beyond this point the weight becomes less than the mean (Text-fig. 1) so that, as a rule, the larger the animal the smaller the mass of organ tissue per unit of body weight that is available to perform a given function. The amount of the reduction in propor-

which is the reverse of that shown by the suprarenals and thyroid. In the case of the hypophysis, both sets of coefficients are large but the negative value is larger than the positive so that the reduction in the weight of the organ per kilo of body weight is more clearly defined than the increase in the actual weight of the organ. The pineal gland exhibits a similar tendency with an even greater discrepancy between positive and negative values. The brain and the superficial lymph nodes are the only other organs that show such a decided preponderance of the inverse over the direct relation between body and organ weight.

As has been pointed out, the parathyroids give results that are more nearly comparable to those of the thymus than to other endocrine glands so far as correlation coefficients are concerned. Both sets of coefficients are small and, while the curves for mean weights are decidedly irregular, neither the actual nor the relative weight of the organs appears to be materially affected by body weight. In fact, if we disregard the upper and lower ends of the curve, the results obtained show the nearest approximation to a neutral equilibrium that is given by any of the organs studied.

The significance of the points brought out by this discussion may be made clearer by tabulating the results for gross body weight in the following manner:

Organ	Coefficient		Organ	Coefficient		Organ	Coefficient	
	Actual	Relative		Actual	Relative		Actual	Relative
Heart.....	+0.755	-0.169	(Testicles)	+0.555	+0.101	Deep		
(Testicles).	+0.555	+0.101	Thyroid..	+0.248	-0.019	cervicals..	+0.246	-0.058
			Supra-			Mesen-		
			renals...	+0.364	-0.066	terics....	+0.307	-0.143
Gastro-								
intestinal								
mass....	+0.456	-0.311	Parathy-					
Kidneys...	+0.471	-0.443	roids....	+0.221	-0.174	Spleen...	+0.186	-0.113
Liver.....	+0.291	-0.301				Thymus...	+0.222	-0.160
Brain.....	+0.451	-0.761	Hypo-					
			physis...	+0.343	-0.402	Popliteals..	+0.190	-0.271
			Pineal...	+0.193	-0.316	Axillaries..	+0.074	-0.321

tion to body weight varies with different organs, but, as a matter of interest, it may be pointed out that, in several instances the correlation coefficients for actual weight give a rough approximation of the part of the organ that is supposed to be essential to the performance of its function, so far as such information is available, or that the coefficient is the reciprocal of the fractional part of the organ or tissue that may be removed without causing serious impairment of function.

Finally, attention should be called to the fact that the results reported above do not represent conditions that obtain in strictly normal rabbits of a given age and breed. They are reported with a realization that not only these but still other factors may have affected the values obtained. There is, however, substantial evidence that the relations found between body and organ weight have an important bearing on the problem of physical constitution and that the results have both an anatomic and a functional significance. In general, it appears that organs that are related anatomically or that may be supposed to perform analogous or related functions give results of a comparable nature, both with respect to the magnitude and the form of the relation shown.

SUMMARY.

Data from 645 normal rabbits were used as the basis of an investigation of the relation existing between body and organ weights. Actual and relative weights were correlated with both gross and net body weight.

The results obtained varied with different classes of organs but it was found that, in general, there was an agreement between the form and degree of the correlation shown and the structural and functional properties of the organs concerned.

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wide variations in weight, the time of occurrence and extent of which are unpredictable and for which the causes are still unknown.

As a means of providing a background for the consideration of more detailed aspects of the problem of the occurrence of variations in organ weight, it seemed desirable to approach the subject with a view to determining first, whether any significant variations in weight of an orderly character could be demonstrated and second, the general course of the variations observed. The purpose of this paper is, therefore, to report the results obtained from a study of the general trend of consecutive variations in mean organ weights of normal male rabbits over a period of $3\frac{1}{2}$ years. The details of the changes observed from month to month and the cause of the variations in weight will not be considered at this time.

Methods and Material.

The material on which this report is based and the methods of investigation that were employed in collecting the data have been described in previous papers (2). We have used results of weight determinations from 645 male rabbits killed in small groups between January 1, 1922, and July 1, 1925. The animals of different groups were fairly comparable as to source, age, and breed; they were given a uniform diet throughout the period covered by the observations but it is not unlikely that the quality of the diet varied and there were some irregularities in the length of time different groups of animals were caged and held under observation before the final weight determinations were made.

The results are presented in the form of a table (Table I) and a series of text-figures (Text-figs. 1 and 2). The actual weights of organs and the weights per kilo of net body weight are recorded in Table I on the basis of the mean values of 39 monthly groups arranged in consecutive order.¹ From these data we have plotted a series of (partially) smoothed curves (Text-figs. 1 and 2) which show the trend of variations in weight from January, 1922, to June, 1925, inclusive, in the form of percentage deviations from the mean normal value. The method of smoothing that was employed is that of a moving weighted average using six monthly groups as the weighting unit according to the following formula:

¹ During the first few months of this investigation, a number of animals that had been used for some minor experimental test was included in the series for normal weight determinations. Subsequently, the data from all of these animals were excluded. In this way all results for July, 1922, were eliminated and the number of animals in other groups was considerably reduced, apparently without materially affecting the results.

THE OCCURRENCE AND TREND OF SPONTANEOUS VARIATIONS IN ORGAN WEIGHTS OF NORMAL RABBITS.

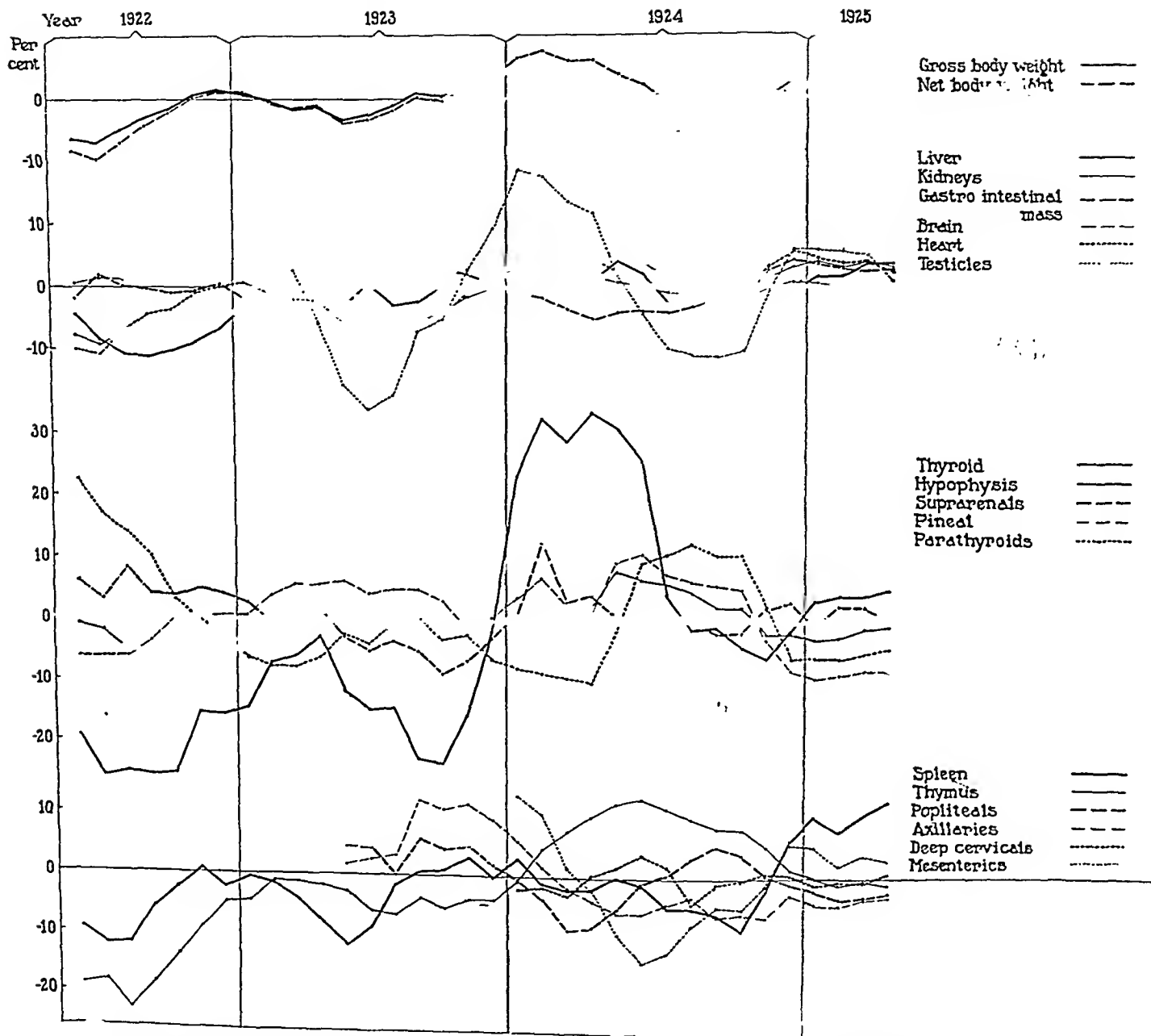
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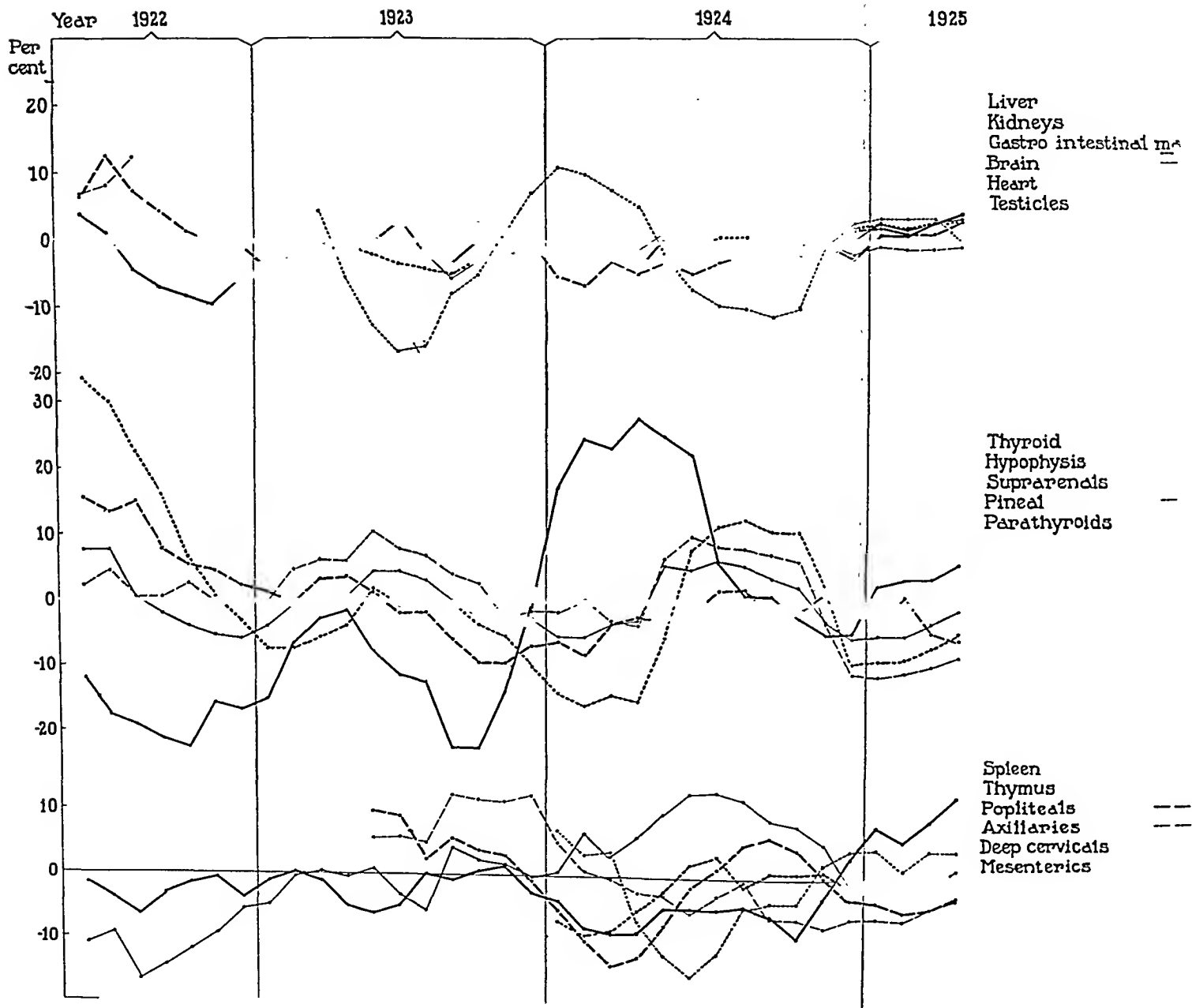
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It is generally recognized that the weights of organs, in proportion to body weight, show wide variations in animals of a given species, that they may differ in the two sexes, and that the ratio of organ to body weight changes with age or development. It has also been found that the weights of some organs, notably the endocrine glands, may be affected by other factors such as dietary and climatic conditions and by sexual activity and that some of these conditions tend to produce periodic or even cyclic changes in weight. These conceptions have been derived chiefly from observations on small groups of animals, studied under various conditions, and, usually, with especial reference to a particular organ or to the influence of some particular condition, so that at the present time there is no clearly defined conception of what this series of variations represents in mature animals of a given sex from the standpoint of consecutive changes affecting the animal organism as a whole over long periods of time. This aspect of the subject of organ weight is, however, of the foremost importance in the study of almost any phase of the problem of physical constitution.

In previous papers (1) we have reported results of weight determinations on a large group of normal rabbits with especial reference to mean values, distribution frequencies, constants which, in general, define the variability of organ weights, the probable limits of variation, and the relations that obtain between the weight of the animal and the weight of organs. Thus far, we have dealt primarily with normal standards but we have emphasized the fact that standard values must be used with extreme caution due to the occurrence of



TEXT-FIG. 1. Variations in body and actual organ weight in terms of the percentage deviation from the mean value.



TEXT-FIG. 2. Variations in the weight of organs per kilo of net body weight in terms of the percentage deviation from the mean.

TABLE I.
Mean Values for Actual and Relative Weights.

Month	No. of animals	Gross body weight	Net body weight	Gastrointestinal mass			Heart			Liver			Kidneys			Spleen		
				Actual		Relative	Actual		Relative	Actual		Relative	Actual		Relative	Actual		Relative
				gm.	gm.		gm.	gm.		gm.	gm.		gm.	gm.		gm.	gm.	
1922																		
January.....	2	2263	1760	503	284	5.08	2.92	106.0	60.6	12.3	6.94	0.640	0.356					
February.....	5	2000	1560	423	272	4.50	2.89	76.6	48.9	10.5	6.75	0.968	0.620					
March.....	6	2408	1918	467	246	5.38	2.81	94.5	50.0	12.8	6.74	1.005	0.535					
April.....	6	2050	1622	413	260	4.66	2.91	81.9	53.1	12.1	7.52	0.810	0.510					
May.....	5	2075	1683	381	227	4.71	2.83	84.2	50.3	12.9	7.65	0.828	0.490					
June.....	12	2050	1654	378	230	4.56	2.75	72.9	44.5	11.6	6.98	1.017	0.621					
September.....	10	2090	1579	502	323	4.63	2.97	71.3	45.9	11.0	6.96	0.752	0.459					
October.....	17	2229	1814	403	223	5.36	2.96	72.0	39.9	12.5	6.91	0.927	0.515					
November.....	14	2464	2025	432	218	5.72	2.85	82.4	41.6	13.0	6.54	1.230	0.620					
December.....	15	2290	1882	403	216	5.09	2.70	83.1	44.4	12.3	6.57	1.070	0.576					
1923																		
January.....	17	2415	1985	417	212	5.69	2.87	84.5	42.8	12.8	6.45	1.105	0.558					
February.....	13	2187	1776	407	232	5.01	2.84	85.8	49.1	12.2	6.99	0.820	0.498					
March.....	15	2088	1675	413	245	5.04	3.02	92.3	56.4	12.3	7.46	0.940	0.577					
April.....	17	2097	1683	406	243	4.81	2.84	76.4	46.3	12.4	7.78	0.876	0.559					
May.....	12	2290	1871	414	220	5.44	2.90	88.1	46.9	12.6	6.78	1.050	0.570					
June.....	9	2347	1950	397	204	4.89	2.51	82.1	42.3	12.2	6.24	0.773	0.388					
July.....	10	2193	1781	412	230	4.71	2.64	86.7	48.1	12.1	6.77	0.941	0.531					
September.....	15	2303	1824	479	269	4.77	2.63	92.7	51.3	12.0	6.69	0.988	0.536					
October.....	14	2234	1809	423	235	5.34	2.92	76.9	43.1	13.0	7.23	1.340	0.735					
November.....	15	2300	1870	427	231	5.12	2.75	77.9	42.5	11.7	6.56	0.976	0.531					
December.....	15	2238	1797	431	243	5.38	3.01	98.0	55.1	13.8	7.77	1.058	0.610					

$\frac{m_1 + m_2 + m_3 + m_4 + m_5 + m_6}{N}$ in which m represents the weighted total for a given month and N the total number of animals for the 6 months period. By this method, major variations are brought out clearly and at the same time some indication of short period variations is retained which we regard as desirable in order to avoid giving an erroneous impression of smooth or perfect continuity.

RESULTS.

The results obtained and the data on which they are based are presented in Text-figs. 1 and 2 and Table I.

DISCUSSION AND CONCLUSIONS.

The results recorded in Table I and Text-figs. 1 and 2 bring out a number of important points concerning the weights of given organs in normal animals, the relation of the weight of one organ to that of others, and the occurrence of variations in weights and relationships. A critical analysis of any of these points would require reference to data which, as yet, have not been presented, but we may refer briefly to a few conditions that are suggested by the smoothed curves in order to clarify certain features of the general tendency that is shown by these curves.

In the first place, there can be no question as to the occurrence of significant variations in the weights of many organs. During the period covered by these observations, it will be noted that all of the organs studied showed apparent variations in weight which assumed the general form of annual cycles. By reference to the tabulated values for mean weight (Table I), it will be seen, however, that the actual variations in weight from month to month were not as uniform as the curves would indicate and that the exact time of occurrence of maximum and minimum weights were in reality somewhat irregular. For example, it is generally stated that the thyroid of animals is largest during the first 4 months of the year, or during the winter, and the curves for thyroid weights would seem to support this view. The figures in Table I show, however, that this is not strictly correct. Exceptionally low values may be obtained at this period of the year and very high values may be obtained for months during which the thyroid is supposed to be small. Perhaps the most constant condition shown by the thyroid, in this case, is the occurrence of low and

TABLE I—Continued.

Month	No. of animals	Thymus		Brain		Testicles		Thyroid		Parathyroids		Hypophysis	
		Actual	Relative	Actual	Relative	Actual	Relative	Actual	Relative	Actual	Relative	Actual	Relative
		gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.
<i>1922</i>													
January.....	2	1.802	1.047	9.63	5.0	6.63	3.75	0.2350	0.1318	0.0200	0.0117	0.0260	0.0149
February.....	5	1.687	1.093	8.54	5.49	5.77	3.69	0.1356	0.0871	0.0134	0.0086	0.0302	0.0195
March.....	6	1.983	1.055	9.32	4.89	6.15	3.18	0.2125	0.1115	0.0173	0.0091	0.0292	0.0154
April.....	6	1.906	1.245	9.47	5.96	6.60	4.05	0.1737	0.1088	0.0168	0.0106	0.0280	0.0176
May.....	5	2.181	1.288	9.31	5.59	4.50	2.64	0.1820	0.1094	0.0134	0.0079	0.0256	0.0152
June.....	12	1.745	1.059	9.49	5.74	4.04	2.47	0.1973	0.1208	0.0159	0.0097	0.0275	0.0167
September.....	10	1.915	1.199	8.60	6.10			0.1306	0.0836	0.0137	0.0088	0.0261	0.0165
October.....	17	1.480	0.824					0.1672	0.0929	0.0132	0.0073	0.0254	0.0141
November.....	14	2.328	1.190					0.1840	0.0936	0.0137	0.0068	0.0271	0.0135
December.....	15	2.414	1.299			6.29	3.52	0.1779	0.0953	0.0111	0.0059	0.0271	0.0146
<i>1923</i>													
January.....	17	2.570	1.324			5.49	2.68	0.2927	0.1465	0.0112	0.0056	0.0274	0.0140
February.....	13	2.423	1.376			4.69	2.62	0.1936	0.1103	0.0132	0.0078	0.0286	0.0163
March.....	15	2.060	1.252			4.61	2.73	0.1681	0.1030	0.0105	0.0063	0.0286	0.0173
April.....	17	1.887	1.132			3.15	1.93	0.2602	0.1504	0.0121	0.0073	0.0276	0.0169
May.....	12	2.313	1.256			4.66	2.47	0.1992	0.1071	0.0143	0.0078	0.0273	0.0147
June.....	9	2.383	1.216			3.65	1.75	0.2174	0.1147	0.0127	0.0065	0.0272	0.0139
July.....	10	2.638	1.481			2.68	1.53	0.1816	0.1026	0.0141	0.0078	0.0301	0.0169
September.....	15	2.006	1.099			3.68	2.03	0.1599	0.0874	0.0122	0.0068	0.0300	0.0165
October.....	14	1.980	1.094			5.23	2.88	0.1683	0.0951	0.0123	0.0069	0.0291	0.0163
November.....	15	2.172	1.736			5.46	2.92	0.1733	0.0924	0.0133	0.0070	0.0269	0.0145
December.....	15	2.088	1.150	9.11	5.37	5.06	2.80	0.1856	0.1047	0.0114	0.0064	0.0261	0.0147

TABLE I—Concluded.

Month	No. of animals	Suprarenals		Pineal		Popliteal lymph nodes		Axillary lymph nodes		Deep cervical lymph nodes		Mesenteric lymph nodes	
		Actual	Relative	Actual	Relative	Actual	Relative	Actual	Relative	Actual	Relative	Actual	Relative
1922													
January.....	2	0.3885	0.2238	0.0120	0.0062							gm.	gm.
February.....	5	0.3228	0.2084	0.0163	0.0107								2.03
March.....	6	0.5193	0.2769	0.0175	0.0092								
April.....	6	0.4165	0.2566	0.0127	0.0080								
May.....	5	0.3556	0.2060	0.0136	0.0081								
June.....	12	0.4032	0.2449	0.0150	0.0093								
September.....	10	0.3545	0.2183	0.0142	0.0091								
October.....	17	0.4366	0.2382	0.0153	0.0085								
November.....	14	0.3868	0.1874	0.0179	0.0089								
December.....	15	0.4020	0.2134	0.0173	0.0094								
1923													
January.....	17	0.4069	0.2057	0.0148	0.0075	0.2700	0.1360	0.1650	0.0830				
February.....	13	0.3816	0.2136	0.0158	0.0091	0.2715	0.1531	0.1790	0.1022				
March.....	15	0.3394	0.2027	0.0148	0.0089	0.3410	0.2065	0.2041	0.1229				
April.....	17	0.3667	0.2194	0.0182	0.0109	0.2440	0.1483	0.1487	0.0897				
May.....	12	0.4392	0.2415	0.0185	0.0099	0.2533	0.1382	0.1929	0.1043				
June.....	9	0.4283	0.2209	0.0177	0.0093	0.2166	0.1109	0.1611	0.0835				
July.....	10	0.2922	0.1589	0.0163	0.0093	0.2552	0.1459	0.1437	0.0818				
September.....	15	0.3333	0.1795	0.0144	0.0080	0.2683	0.1472	0.1863	0.1022				
October.....	14	0.3740	0.2056	0.0152	0.0085	0.2850	0.1599	0.2124	0.1206	0.2092	0.1198	3.48	1.95
November.....	15	0.3357	0.1784	0.0183	0.0099	0.3177	0.1714	0.2230	0.1229	0.1510	0.0789	3.81	1.95
December.....	15	0.3507	0.1943	0.0165	0.0091	0.2355	0.1272	0.1787	0.1013	0.1105	0.0666	4.03	2.23

comparatively uniform values during the months of September, October, and November, and even in December, rather than the occurrence of high values from January or December to April inclusive. Moreover, it will be seen that the thyroid showed a late spring or early summer increase in weight with as much regularity as it did the more generally recognized winter increase which, after all, may be comparatively slight and of irregular occurrence and duration, with intervening periods during which the weight of the organ is small.

These conditions are mentioned in order to make it clear that, while there is an evident tendency to the occurrence of cyclic variations in the weights of many organs with distinct seasonal relations, these changes do not occur with perfect regularity or in perfect conformity with the (astronomical) progression of the seasons. At times, the departure from the usual course is very decided.

A second point to be noted is the variation in the magnitude of the change from year to year. This is again illustrated best by the thyroid which shows that, in addition to the annual cycle, there was a progressive increase in the magnitude of the values obtained over a period of at least 3 years. Scattered observations made during the latter part of 1921, which are not included in the results recorded above, indicate that the maximum weight of the thyroid for the winter of 1921-22 was approximately the same as that given for the winter of 1922-23; it may have been a little higher or a little lower. During the winter of 1924-25, the weight was lower than for the preceding winter but it was still high (see Table I) and, while systematic observations were discontinued in June, 1925, sufficient data have been accumulated to make it certain that the weight of the thyroid increased during 1925-26 to a much higher level than any hitherto recorded by us. This applies to rabbits raised in our own laboratories as well as to those obtained from the usual outside sources which shows a widespread prevalence of a condition that might even be termed a goitrous enlargement.

If all of these observations are taken into account, the results obtained suggest the occurrence of a second cycle of weight variation extending over a period of years. The indications are that our observations may have been started at or near a period of minimum

1924													
January.....	18	0.4277	0.2051	0.0139	0.0069	0.2492	0.1228	0.1819	0.0924	0.1353	0.0671	4.48	2.24
February.....	17	0.3996	0.2008	0.0175	0.0095	0.2173	0.1158	0.1394	0.0965	0.1206	0.0623	4.08	2.14
March.....	20	0.4128	0.1904	0.0174	0.0081	0.2368	0.1099	0.1573	0.0749	0.1894	0.0834	3.62	1.68
April.....	15	0.3653	0.1873	0.0182	0.0095	0.2255	0.1191	0.1619	0.0853	0.2100	0.1050	2.80	1.46
May.....	22	0.4000	0.2309	0.0145	0.0083	0.2325	0.1316	0.1782	0.1084	0.1414	0.0812	2.35	1.36
June.....	14	0.3759	0.2115	0.0151	0.0087	0.2484	0.1424	0.1529	0.0873	0.1493	0.0863	3.16	1.79
July.....	19	0.3545	0.2018	0.0214	0.0119	0.2927	0.1628	0.1632	0.0909	0.1482	0.0826	2.97	1.65
August.....	20	0.3779	0.2134	0.0186	0.0111	0.2835	0.1635	0.1422	0.0813	0.1443	0.0828	3.00	1.72
September.....	32	0.4236	0.2233	0.0154	0.0082	0.2547	0.1374	0.1682	0.0892	0.1680	0.0894	3.61	1.94
October.....	15	0.3263	0.1792	0.0166	0.0093	0.2823	0.1583	0.1787	0.0991	0.1323	0.0729	4.09	2.30
November.....	19	0.3521	0.1934	0.0139	0.0076	0.2591	0.1401	0.1484	0.0798	0.1732	0.0925	2.92	1.56
December.....	14	0.3789	0.2074	0.0144	0.0080	0.2214	0.1216	0.1568	0.0862	0.1546	0.0853	3.12	1.72
1925													
January.....	45	0.4143	0.2231	0.0144	0.0078	0.2486	0.1350	0.1580	0.0855	0.1585	0.0856	3.82	2.10
February.....	40	0.3983	0.2003	0.0138	0.0070	0.2550	0.1313	0.1763	0.0889	0.1492	0.0766	3.88	1.99
March.....	27	0.3399	0.1945	0.0144	0.0082	0.2352	0.1365	0.1594	0.0925	0.1546	0.0875	3.46	2.00
April.....	30	0.4283	0.2348	0.0159	0.0088	0.2498	0.1365	0.1693	0.0937	0.1497	0.0815	3.27	1.79
May.....	20	0.3500	0.1962	0.0150	0.0085	0.2665	0.1475	0.1663	0.0930	0.1600	0.0950	3.54	1.98
June.....	14	0.2736	0.1794	0.0142	0.0092	0.2366	0.1526	0.1605	0.1017	0.1782	0.1130	2.76	1.80

The changes exhibited by the heart, brain, kidneys, liver, and gastrointestinal mass are of an entirely different order from those of the endocrine glands, the testicles, and lymphoid tissues. This raises the question of the significance of the variations shown and, while it is not our intention to enter into a general discussion of this aspect of the subject at the present time, it seems desirable to refer briefly to the possible influence on organ weight of certain factors that are inherent in the material, namely, the age and weight of animals.

The curves for gross and net body weight in Text-fig. 1 show periodic variations similar to those of the curves for organ weight. The variations in these curves represent not only differences in body weight but differences in age as well and are due to unavoidable conditions of animal supply. For example, during the winter months the supply of fully mature animals is more abundant than at any other time; as the supply of such animals becomes depleted, it is necessary to use a larger proportion of younger and smaller animals during the spring, summer, and early fall. These conditions are repeated year after year; they are reflected in the body weight curves and undoubtedly do have an effect on the curves for organ weight.

The factors of age and weight are so intimately bound up with each other that we cannot attempt to make any distinctions at this time but some indication of the influence that might be expected from this combination of factors may be obtained from the correlation coefficients for organ and body weight (3) which were calculated without distinction as to age so that they are directly applicable to the present instance. On this basis, one would expect that the body weight factor would affect the weights of the heart, testicles, kidneys, gastrointestinal mass, and brain more than other organs as their correlation coefficients are larger, and a careful comparison of the curves will show that the curves for actual and relative weights of these organs do conform most closely with those for body weight. Moreover, the degree of conformity is roughly proportional to the magnitude of the correlation coefficients. The gastrointestinal mass forms an exception and the extent of the variation shown by the testicles is out of proportion to that of other organs in this group.

It is evident, therefore, that, in the case of the organs mentioned, the body weight factor is of considerable importance and may indeed

thyroid weight (a period of maximum weight for some organs) or at the turning point of a decreasing phase of a cycle and that most of our observations fell within the limits of an increasing phase, the maximum of which we are now approaching or have reached during the past few months.

A third feature of the results to which attention may be directed is the suggested relation of the change in the weight of one organ to that of other organs. This is also shown best by the relation of the curves for the thyroid to those for other members of the endocrine system but it will be seen that still other organs show something of the same relation to the thyroid. In general, it may be said that the weight curve for the thyroid stands alone while those for the other endocrines tend to group themselves together and to move in a direction opposite to that of the thyroid with a definite lag in the movements. Still, these relations are not entirely constant. At times, all of the curves are more or less parallel and move in the same direction or they display irregularities in their movements. There is, however, a definite suggestion of a coordination of movements and apparently the manner in which this coordination is effected is influenced to some extent by the actual as well as the relative positions of the organs with respect to the mean normal or zero line. Thus, during 1922 and the early part of 1923 there was an apparent tendency for the weights of all organs to converge toward the mean normal. For a time thereafter, weights were comparatively stable while movements were more or less parallel and in the same direction. But, with the subsequent marked increase in the weight of the thyroid, the curves for other organs continued their downward course, initiating a series of reverse movements which were very decided and clearly defined during the greater part of 1924. This series of changes was followed in time by a second period of comparative stability and parallel coordination of movements which differed in some respects from that of 1923. This apparent variation in the coordination of weights at different levels with respect to the mean normal illustrates the difficulty of attempting to deduce a general rule governing the relation of one organ to another.

Finally, attention may be called to the great difference in the magnitude of the variation in weight shown by different organs.

the weights of organs that must occur from time to time in order to maintain the health of the animal under changing conditions of life. Hence, weights and relations that would be regarded as normal for one period might be decidedly abnormal for another. Recognition of the occurrence of variations in weight as an essential attribute of organs and a knowledge of the extent to which the weights of various organs may be affected by environmental conditions is of fundamental importance in the study of problems of physical constitution from either an anatomic or functional point of view and is deserving of especial emphasis as the tendency at the present time is to stress standardization and conformity to rule rather than the occurrence of variations.

SUMMARY.

Records of organ weights of normal rabbits covering a period of $3\frac{1}{2}$ years were analyzed with a view to determining first, whether any significant variations of an orderly character could be demonstrated and, second, the general trend of the variations that occurred.

It was found that many organs showed decided variations in weight which assumed the form of definite annual cycles. In addition, there was some evidence of a second cycle covering a period of years. These changes were most marked in the case of the endocrine glands (including the testicles), the lymphoid organs, and the liver.

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account for the greater part of the variation shown. The liver appears to be less affected by this factor and a comparison of the curves for the endocrine glands and lymphoid tissues with those for body weight shows that the magnitude of the values and the movements of the curves are virtually independent of those for body weight. Still, it must not be assumed that the body weight factor had no influence on the results obtained for these organs as some of them show a fairly close correlation with body weight. On the whole, the results are in agreement with what might be expected on the basis of the correlation coefficients and it seems that, in the case of these organs, the influence of body weight was of subsidiary importance. There is some evidence, however, that, at times, the influence of this factor may have been such as to alter or mask the effects of other conditions and this possibility should not be overlooked.

It would appear, therefore, that the results recorded above give a composite picture of variations in weight due largely to external causes. At the present time, we are not concerned with point to point variations or with the exact magnitude of the change in weight shown but with the general course of events and the occurrence of changes that are so clearly defined and of such proportions that their significance cannot be questioned. The course of events represented by this series of observations may not agree in all respects with what might have been found had it been possible to follow the weights of organs in a given group of animals over the same period of time, but the manner in which the investigation was carried out and the nature of the results obtained are sufficient to warrant the conclusion that the picture presented gives a fair conception of changes in weight and weight relationships that actually occurred at this particular time.

If we consider these results from the point of view of normal standards, it is at once apparent that no fixed standard of weight or relation can be established. Conditions that prevail at one time differ greatly from those found at another and thus far we have no acceptable basis for making discriminations as to the normality of the findings for one period as compared with another. It seems that the animal organism is subject to the influence of extraneous conditions which determine the relations that obtain and the changes in

agglutinin has been shown to be identical with that of globulin, pH 5.2-5.4 (3, 4). This has been disputed recently (5), but our own experiments support this conclusion. Agglutinin is not part of the serum albumin (2) and is not removed by lipid extraction of the serum (6). That agglutinins are globulins is still unsettled; but that the agglutinin-globulin complex is the essential reacting substance seems clear.

Very important advances have been made recently by Avery, Heidelberger, and their coworkers (7) and Zinsser, Mueller, *et al.* (8), in the direction of ascertaining the chemical constitution of the portions of the bacterial cell which take part in specific interactions with antibody. The first group of workers have isolated from the three fixed types of pneumococcus and from strains of *B. friedländeri*, certain chemically distinct substances which they have shown to be polysaccharides, which react specifically with homologous antisera. These authors believe that these type-specific substances are to be found in the ectoplasmic layers of the cell and that, in view of the fact that the immune reactions are presumably cell surface phenomena, the presence in the periphery of the organisms of these substances determines the specificity and the readiness of response of the reaction. These workers have also isolated less clearly defined substances (nucleoproteins) which they have shown to be specific species-reacting substances. The second named investigators have shown that certain non-protein residue substances (carbohydrate gums) may be extracted from many types of bacteria and from yeasts, and that they bear a definite relation to the specific character of the organisms in that they react specifically with homologous immune sera.

To explain the mechanism of the union between antibody and organism, two main hypotheses have been suggested. The first, that the combination of the two is due to their possessing opposite electrical charges, seems to have been disposed of finally by Northrop and De Kruif (9) who have shown that union may occur when both elements are similarly charged.

The second explanation assumes that the antibody forms a film at the surface of the organisms and is held by ordinary forces of valence. A counterpart of this is found in the observation of Loeb (10) that collodion particles treated with proteins acquire a firmly fixed film of protein at their surfaces; a corollary, of much importance, in his work, is the observation that such film formation causes the particles to assume certain characteristics, notably the cataphoretic behaviour, of the proteins used.

In favour of the second hypothesis may be cited the following. Coulter (11) has shown that the optimum pH for agglutination of red blood cells is 4.75, but that when they are sensitized the optimum shifts to pH 5.3, *i.e.*, the isoelectric point of serum globulin. That is, in respect of their acid agglutination point, the red cells now behave like particles of euglobulin. Similar findings have been reported by Northrop and De Kruif (12) in experiments with bacteria and normal and immune sera, egg albumin and globin, and by Eggerth and Bellows (13) with bacteria and various proteins. That this assumption is correct seems to be established by certain of our experimental results that appear below.

STUDIES IN AGGLUTINATION.

III. ON THE MECHANISM OF THE AGGLUTINATION OF BACTERIA BY SPECIFIC AGGLUTINATING SERUM.

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INTRODUCTION.

The first hypotheses concerning the laws governing immunological reactions were characterized by considerable complexity. The effort to describe these phenomena in chemical-physical terms has led to much clarification. In the reaction of bacterial agglutination, in particular, this type of approach has been most productive, and the earlier hypothetical explanations of the reaction have been gradually replaced by conceptions which conform with general chemical-physical laws.

Very early in the study of the phenomenon of specific bacterial agglutination, Bordet (1) made the observation that when bacteria are treated with homologous agglutinin, no flocculation is possible until electrolytes have been added. He thus conceived of specific agglutination as occurring in two phases, *first*, sensitization of bacteria by agglutinating serum (union of antibody and antigen) and *second*, flocculation of the agglutinin-bacteria complex by the electrolytes of the suspending fluid. He considered this flocculation analogous to the precipitation of colloidal suspensions by salts. That this two-phase conception is essentially correct has been abundantly confirmed.

First Phase.—Little is known of the reason for the selectivity of the interaction between agglutinin and homologous organism, *i.e.*, specific sensitization. The explanation will probably be forthcoming only when the exact chemical nature of the two reacting substances is determined.

The general character of agglutinin is known. It has been shown to be closely associated with the serum globulin, as it is precipitated out of the serum in the globulin fraction, and remains with the globulin (either euglobulin, pseudoglobulin, or both (2)) after purification of the protein. Also, the isoelectric point of the

Their conclusions may be summarized briefly as follows: (1) Agglutination is to be considered in terms of two antagonistic forces: a *repelling force*, due to like electrical charge, which tends to keep the bacteria apart, and "*cohesive force*," which makes for adhesion. In any bacterial suspension, all factors that make the repelling force relatively greater than the cohesive force make for stability; and conversely, all factors that reduce the repelling force or otherwise make the cohesive force relatively greater, lead to flocculation. (2) In the case of unsensitized bacteria, electrolytes in lower concentrations, < 0.01 N, affect primarily the potential, and in higher concentrations, > 0.01 – 0.1 N, affect primarily the cohesive force. (3) As long as cohesive force is unaffected, agglutination occurs whenever the charge is reduced by electrolytes to a point below a critical level of about 15 millivolts;² that is, the unaffected cohesive force now becomes relatively greater than the force of repulsion. (4) Salt in high concentration depresses the cohesive force of unsensitized bacteria so that no agglutination occurs even though there may be no measurable charge; *i.e.*, the cohesive force is now so small that it is always less than the repelling force. (5) When bacteria are treated with immune sera, their cohesive force is in some manner protected from this depressing effect of strong salt (*e.g.*, physiological salt solution, etc.) and agglutination is determined solely by the charge; that is, whenever the potential of the sensitized bacteria is reduced by electrolyte to a point below 15 millivolts, the suspension agglutinates. This explains the observation of Bordet, confirmed by Northrop and De Kruif in the course of the work being cited, that electrolytes are essential for specific agglutination. That is to say, the salt, routinely used in the ordinary reactions, reduces the charge on the bacteria so that this charge comes to lie in the 15 millivolt agglutination zone, and, the cohesive force of the sensitized bacteria, being insusceptible to the depressing effect of the electrolyte, is now relatively greater than the repelling force and flocculation occurs.³ (6) Their results refute the idea that combination of antibody and organism is caused by difference of sign of the charges carried by the two substances; but are in agreement with the assumption that the agglutinin forms a film on the surface of the organism.

In the course of studies concerned with the importance of environmental factors in agglutination (23), we have been much interested in the nature of the changes in electrical charge that occur when bacteria are treated with agglutinative sera. As a result of observations upon these changes, much light has been shed upon the general nature

² Loeb (22) has shown that a similar critical potential agglutinating zone exists for many varieties of suspended particles, *cf.*, native and denatured proteins, collodion, graphite, etc.

³ That this may not be the only part played by electrolytes is indicated, however, in experiments still under investigation, with organisms having a low primary charge, less than 15 millivolts.

Second Phase.—Much more is known of this aspect of the problem. Bechold (14), Neisser and Friedemann (15), and Arkwright (16), in support of Bordet, have emphasized the similarity between bacterial and colloidal suspensions in demonstrating the fact that bacteria possess an electrical charge, as evidenced by migration in an electrical field.¹ The two first named authors have carried the analogy further by demonstrating the similarity between the salt precipitation of kaolin and mastic, and of sensitized bacteria. They also have stated that sensitized bacteria lose their charge and have suggested that neutralization of charge is responsible for agglutination, recalling the observation of Hardy that suspensions of denatured protein (egg white) coagulate at the point at which they are devoid of charge; that this last assumption is partially incorrect appears later.

Tulloch (17) observed that, in respect of their behaviour toward electrolytes, unsensitized bacteria resemble fresh egg white and sensitized organisms resemble denatured egg white, *i.e.*, the former are precipitated by strong concentrations of salt only, while the latter flocculate readily in low salt, *e.g.*, physiological salt solution. He concludes that the process of sensitization is akin to denaturation of bacteria.

Buchanan (20), in an exhaustive review of the subject of agglutination, suggests that the like charges on bacteria act as forces of repulsion to keep the suspensions dispersed and that the force of surface tension acts as the attracting agency to cause agglutination. He suggests that agglutination occurs whenever factors are introduced which make the repelling force relatively less than the pull of surface tension.

The most valuable contribution to our knowledge of the chemical-physical laws governing bacterial agglutination is found in the recent work of Northrop and De Kruif (9, 12, 21). Their findings are so important, and so satisfactorily correlate the observations of previous investigators, that those conclusions bearing specifically upon the present consideration may well be reviewed at considerable length.

These authors accept the hypothesis that there are two factors making for repulsion, or for adhesion (and subsequent clumping) of bacterial particles; the first being the electrical charge on the organisms, and the second, a force, still insufficiently defined, to which they have assigned the name "cohesive force." They assume that, whenever the latter force is relatively higher than the former, agglutination results. However, they maintain that no satisfactory testing of these hypotheses is possible without measurement of both forces; accordingly they have devised a technique for measuring cohesive force. The method, as they indicate, is not one of absolute precision, but the values obtained are regularly reproducible; and in our hands (23) the method has given perfectly satisfactory results. Bacterial charge was measured from the rate of migration in a cataphoretic cell.

¹ For theoretical discussions of the origin of this charge see the work of Loeb (10, 18), Northrop and De Kruif (21), and Winslow, Falk, and Caulfield (19).

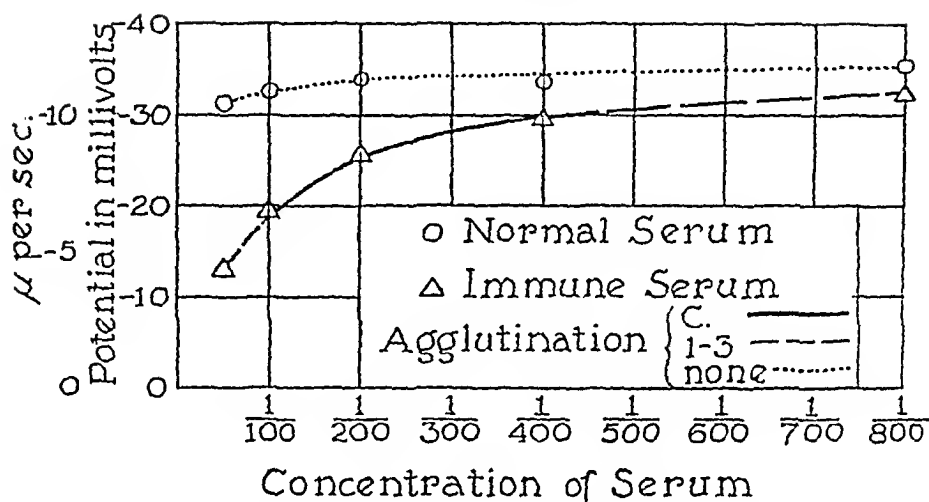


FIG. 1. Effect of normal serum and Type I pneumococcus serum on potential and agglutination of Type I pneumococcus. Experiment in $M/200$ glycoll-phosphate-acetate buffer, pH 7.0. In this and subsequent figures complete agglutination is shown by a solid line, incomplete by a broken line, and absent agglutination by a dotted line. This figure is kindly reprinted from the previous paper (24).

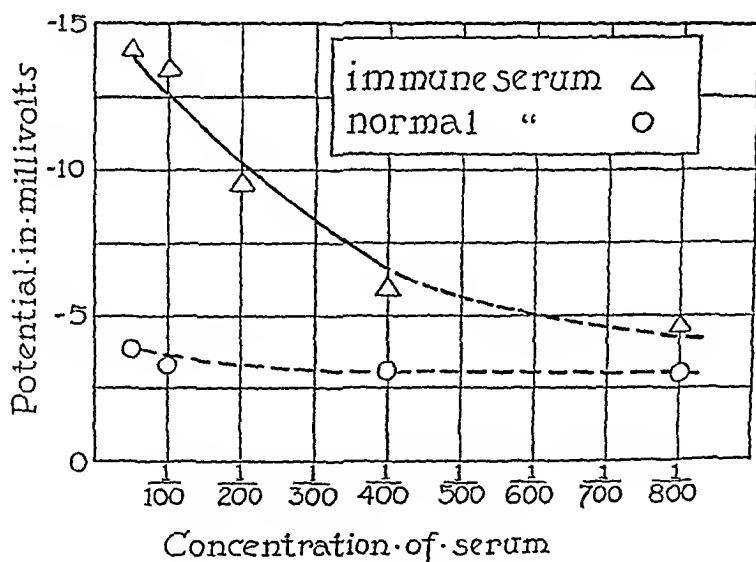


FIG. 2. Effect of normal serum and specific agglutinative serum upon *B. dysenteriae*, Shiga strain. There was no agglutination by normal serum. Experiment in $M/200$ glycoll-phosphate-acetate buffer, pH 7.0.

of the reaction, and we now feel that we have sufficient data upon which to found a satisfactory hypothesis for the mechanism of the agglutination of bacteria by specific serum.

Methods.

Pseudoglobulin was obtained from human ascitic fluid as follows: the globulin was thrown down with 50 per cent $(\text{NH}_4)_2\text{SO}_4$ and the precipitate put into membranes and dialyzed against running tap water 48 hours. To this, $(\text{NH}_4)_2\text{SO}_4$ to make 25 per cent, was added to remove the euglobulin—to the filtrate enough salt was added to make 50 per cent and the pseudoglobulin precipitate was redialyzed against tap and distilled water at pH 5.4. The precipitate in this was discarded. The resulting solution of pseudoglobulin was 4.5 per cent.

All other methods used, unless otherwise indicated, are similar to those described in the preceding paper (24).

EXPERIMENTAL RESULTS.

The first step in specific bacterial agglutination is selective interaction between agglutinin and organism. As will be recalled, the evidence thus far obtained favours the assumption that this union consists of a coating of the organism by agglutinin. Proof of the validity of this assumption is essential to the hypothesis for the mechanism of the reaction to be outlined below. The following experiments seem to establish the correctness of this conception of sensitization.

In a preceding paper (24), it has been shown that in the cases of Type I pneumococcus and *B. paratyphosus* A, their agglutinative sera possess a specific charge-reducing effect which is quantitatively related to the titer of the serum. It has been shown that this effect disappears following absorption of agglutinin, and that it is not present in highly protective sera that have no agglutinative properties.⁴

Fig. 1 illustrates this effect in the case of the Type I pneumococcus serum. It will be seen from this figure that both normal and immune sera depress the charge on the bacteria as their concentration is increased, but it will be observed that the normal serum effect is only slight while the reducing effect of the specific serum is striking.

⁴ We have since observed also that the protective antibody of Huntoon (25), which is practically non-agglutinative, does not show this specific effect.

found that the potential agglutination zone of unsensitized cells is low (below 6 millivolts) and that, when sensitized the critical point is raised to about 12 millivolts. This is approximately the critical point for globulins, as will be seen below. Accordingly, the difference between the unsensitized and the sensitized cells may well be accounted for on the assumption that the latter are coated with specific antibody (globulin).

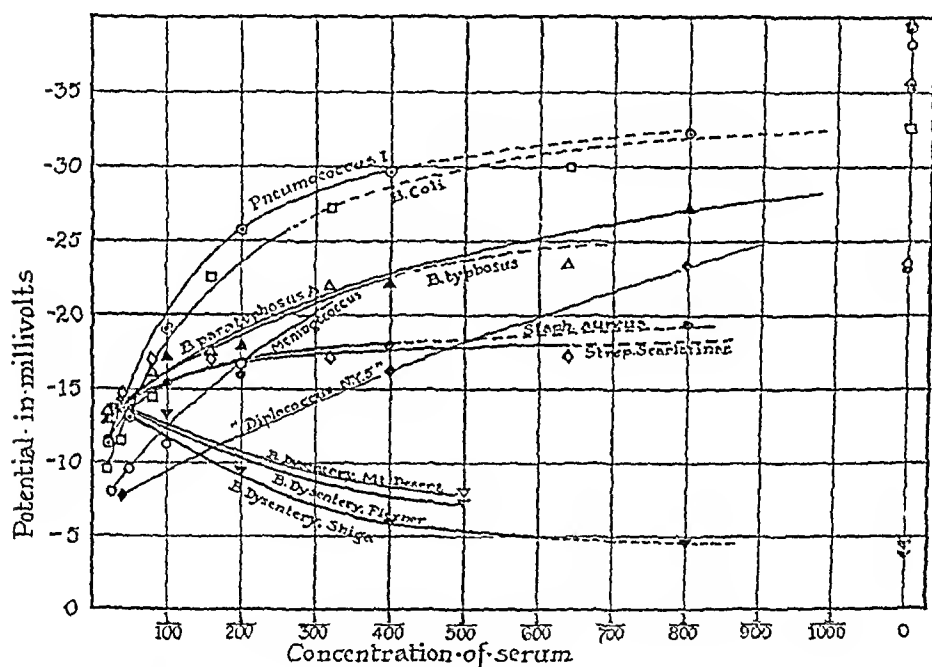


FIG. 3. Effect of eleven specific agglutinating sera upon the potential and agglutination of their homologous organisms. These experiments were done over a period of about 2 years and three different cataphoretic cells were utilized in the work. All experiments in $M/200$ glycoll-phosphate-acetate buffer mixture, pH 7.0.

That the conception of sensitization as specific coating with globulin is correct is made still more clear by the evidence afforded in the following experiments.

The cataphoretic behaviour of sensitized Type I pneumococcus was compared with that of the globulin from the homologous serum as follows: The euglobulin of the serum was precipitated by 1:20 dilution with distilled water. The particles thus obtained have protective

We have since extended observations of this phenomenon to several other bacteria and their agglutinating sera. The organisms studied now include, Type I pneumococcus, meningococcus, staphylococcus, *Streptococcus scarlatinae*, a diplococcus variant of a hemolytic streptococcus, *B. coli*, *B. typhosus*, *B. paratyphosus* A, *B. tuberculosis*, and three strains of the dysentery bacillus, Flexner, Shiga, and Mt. Desert.

Confirmation of the fact that agglutinative sera possess this charge-reducing effect was obtained in all cases studied except the three dysentery strains. In the case of these organisms, an unexpected result was secured when it was found that their specific sera in high concentration not only do not reduce the charge, but raise it. Fig. 2 illustrates this effect in the case of the Shiga strain and its agglutinative serum.

When the specific effects of all the agglutinative sera upon the cataphoretic charges of their homologous organisms are plotted upon the same chart (Fig. 3), the explanation of this paradoxical behaviour becomes evident. Reference to this figure brings out the fact that all the organisms other than the dysentery strains have, under the conditions of the experiment, high or relatively high charges (23 to 40 millivolts) when untreated with serum; while the three dysentery strains possess relatively low charges (3 to 5 millivolts). It will be seen that in the case of the first group cited there is specific charge reduction, quantitatively related to the agglutinative titer, and that in the second group there is a similar charge elevation. In all cases, however, no matter what the initial charge (range is 5-40 millivolts) high concentrations of serum (over 1:50), bring the charges on all bacteria to a *common potential level* (8-14 millivolts).

In other words, when different bacteria are treated with homologous agglutinative sera in high concentration, they behave cataphoretically alike, irrespective of their previous differences in charge. This suggests that they are alike because they have a similar surface coating. To put it in another way, when bacteria are sensitized by agglutinative sera, a film of agglutinin (globulin) is formed on their surfaces and such sensitized bacteria, in the sense of Loeb's protein-coated particles discussed above, all behave cataphoretically like particles of globulin.

It is worth calling attention, at this point, to the work of Northrop and Freund (26) on the agglutination of red blood cells. They have

of varying hydrogen ion concentration. The bacteria were colon bacilli treated with 1:20 homologous agglutinating serum, centrifuged, and then resuspended in the solutions in which they were to be tested. The pseudoglobulin, non-specific, was denatured by bringing it, in dilute solution, to the boiling point; the resultant flocculant mass was centrifuged, divided into small homogeneous particles in a mortar, and added to the solutions of varying pH. Unsensitized colon bacilli were tested similarly for comparison. The results appear in Fig. 5.

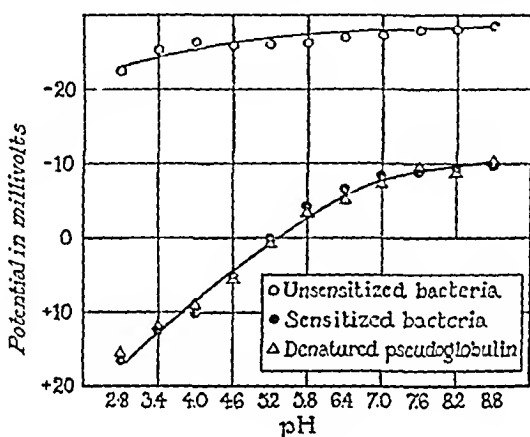


FIG. 5. Effect of varying pH upon potential of unsensitized and sensitized colon bacilli and of particles of denatured pseudoglobulin. Experiment with glycocoll-phosphate-acetate buffer, $M/25$. Agglutination of sensitized bacteria and pseudoglobulin was complete throughout and was absent in case of unsensitized colon bacilli.

It will be observed that the cataphoretic behaviour of the sensitized bacteria is quite unlike that of the unsensitized organisms, but it is practically identical with that of the denatured pseudoglobulin particles. In other words, the sensitized organisms behave cataphoretically as if they are particles of globulin. A finding again accounted for by the assumption that the organisms are coated with agglutinin.

It will be noted also, from this chart, that the isoelectric point of the sensitized bacteria is the same as that of the pseudoglobulin, which is the accepted one of approximately pH 5.3. This is in accord with the findings of Coulter, Northrop and De Kruif, and Eggerth and Bellows, already cited.

antibody (Felton (27)), and agglutinin, as demonstrated by control experiments; they are readily visible microscopically and are thus susceptible of study in an electrophoretic cell. The bacteria were sensitized by treatment with 1:20 immune serum. Unsensitized organisms and organisms treated with 1:20 normal serum were used as controls. The four sets of particles were now added to varying concentrations (M/25-M/800) of glycoll-phosphate-acetate buffer (21) of pH 7.0, and their charges determined. The results are shown in Fig. 4. The upper curve is that of the untreated organisms, and

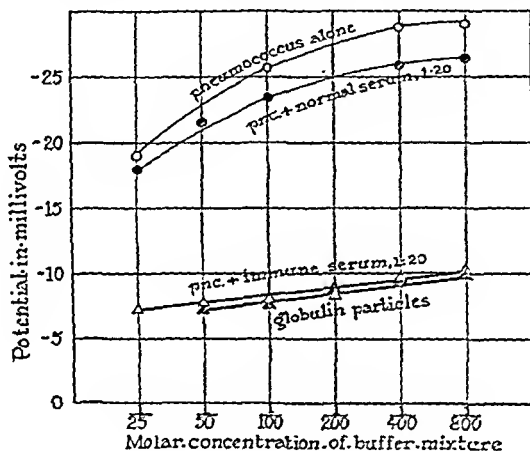


FIG. 4. Effect of glycoll-phosphate-acetate buffer, pH 7.0, in varying molar concentration, upon potential of *Pneumococcus* Type I, (a) alone, (b) treated with 1/20 normal and (c) with 1/20 specific serum, and (d) of globulin particles obtained from the specific serum.

the next, that of those treated with normal serum, showing the usual slight charge reduction. The two lower curves are those with which we are chiefly concerned. It will be seen that the curve of the sensitized organisms is practically identical with that of the globulin particles. That is, the former are acting cataphoretically as if they were globulin, a fact which can be explained only on the assumption that they are coated with globulin.

The cataphoretic charges of sensitized organisms were next compared with those of particles of denatured pseudoglobulin in solutions

as soon as their charge is reduced by electrolyte to a critical point of about 13 millivolts.

It is important to note, that in the case of particles of denatured protein, the cohesive force (in the sense used for bacteria, by Northrop and De Kruif) is practically insusceptible to the reduction effect of high concentrations of salt. This is true also for protein-coated particles.

These findings of Loeb supply the reason for the observed fact that sensitized bacteria behave like particles of denatured protein. That is, when the organism is coated with agglutinin and assumes the character of a globulin particle, by virtue of this very film formation, it takes on the character of denatured globulin. Because of this similarity, Tulloch, as noted above, believed that sensitization is akin to denaturation of bacteria. However, the bacterial surface itself is not "denatured," but the film of globulin formed at its surface assumes the characteristics of denatured protein, and the coated organism now acts like a particle of denatured globulin. The same author's analogy between native protein and unsensitized organisms, also noted above, is probably incorrect, as the behaviour of the former is dependent upon true solubility and the failure of the latter to flocculate is due to reduced cohesive force, as shown by Northrop and De Kruif.

The observation of Northrop and De Kruif that the cohesive force of sensitized bacteria is protected against reduction by strong salt is also accounted for. The explanation lies in the fact that the surface film of globulin, having the character of denatured protein, is insusceptible to this depressing effect, and that while electrolytes may reduce the charge on the coated particles, they do not affect their cohesive force, and agglutination is determined solely by reduction of the potential to the critical 15 millivolt level.

Before proceeding to full application of these findings of Loeb to specific agglutination, it seemed necessary to prove that the behaviour of globulins with collodion particles is similar to that of the proteins used by Loeb. Accordingly, certain of his experiments were repeated with pseudoglobulin.

The procedure followed was essentially that described by Loeb (10, 18, 22). Collodion particles were coated with isoelectric pseudoglobulin. Particles of denatured pseudoglobulin were prepared by bringing a dilute solution to the boiling point, centrifuging the floc-

The evidence presented above seems to lead reasonably safely to the conclusion that the first step in specific bacterial agglutination is a selective film formation at the surface of the organism by the globulin of the antibody, and that the changes in charge on the bacteria are the result of this coating. In high concentrations of homologous agglutinating serum, the coating by globulin is complete and the organisms behave exactly like particles of globulin. When the serum is dilute, the degree of coating is less and the charges observed represent the potential difference between the surrounding medium and a combination of bacterial surface and agglutinin film.

What degree of coating is essential for agglutination of the bacteria we are not prepared to say, as the data for such estimation are difficult to obtain. Northrop and De Kruif believed from their experiments, that if film formation could be assumed, it could be calculated that agglutination occurs when the surface is one-eighth covered. From examination of our results with sera having a high agglutinative titer, it is our impression that the amount of coating required may well be less than this.

The further interpretation of the part played by this selective coating of the bacteria in specific agglutination is based largely upon certain observations and conclusions of Loeb (10, 18, 22) in his work with collodion particles and proteins. It is necessary therefore, to consider briefly those portions of his work that bear upon our problem.

Loeb treated collodion particles with various proteins, egg albumin, gelatin, casein, edestin, and found that a durable film of these proteins is formed at the surface of the particles. He showed that such coated particles now behave cataphoretically as if they are no longer collodion particles but are particles of the protein used.

He observed further, that in respect of their stability in suspension, the coated particles (except those treated with gelatin) no longer behave like soluble native proteins which are precipitated only by very strong salt, but act like denatured proteins, which are relatively unstable and are easily thrown out of suspension by electrolytes in low concentration. That is to say, the chemical forces of attraction between the "aqueous" groups of the protein molecule and the water are destroyed in the film formation, as is the case when protein is denatured by boiling, and the stability of the coated particles depends only, as does that of denatured proteins, upon the electrical double layer surrounding each particle (*i.e.*, the charge). To recapitulate, collodion particles coated with protein no longer act like collodion particles, but behave as if they are particles of denatured protein, and flocculate

SUMMARY.

From the foregoing evidence, specific bacterial agglutination may be conceived of as follows: When bacteria are mixed with their homologous agglutinative sera, specific union between organism and agglutinin occurs. This interaction consists of specific coating of the bacteria by globulin. By virtue of the fact, noted by Loeb and illustrated in Fig. 6, that protein film formation gives the coated particle the characteristics of denatured protein, the bacteria now take on the character of particles of denatured globulin. Particles of denatured protein flocculate whenever their charge is reduced by electrolyte to a critical level lying somewhere between 12 and 14 millivolts. This is true even when the salt is strong, as, unlike bacterial particles, their cohesive force is not readily depressed by salt. The sensitized bacteria, now being essentially particles of denatured protein, likewise agglutinate as soon as their charge is reduced by electrolyte to this potential level.

CONCLUSIONS.

1. In the process of sensitization by agglutinating serum bacteria are coated selectively by the globulin of the antibody.
2. This film formation causes the bacteria to take on the characteristics of particles of denatured globulin.
3. Subsequent agglutination of the coated bacteria follows the laws governing the flocculation of particles of denatured protein by electrolytes.

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culant mass, and dividing it into suitable homogeneous particles in a mortar.⁵ The effect of three electrolytes, NaCl, $K_4Fe(CN)_6$, and $CeCl_3$, upon the charge and agglutination of untreated collodion particles, coated particles, and particles of denatured pseudoglobulin was determined at pH 3.0.⁵ The results, shown in Fig. 6, demonstrate the fact that pseudoglobulin behaves in similar fashion to the proteins used by Loeb. Reference to the figure shows that collodion particles treated with pseudoglobulins no longer behave like the untreated

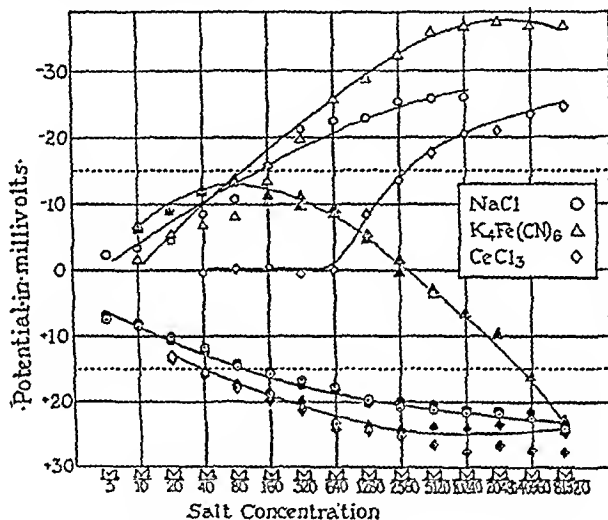


FIG. 6. Influence of NaCl, $K_4Fe(CN)_6$, and $CeCl_3$ upon potential and agglutination of collodion particles (blank symbol), collodion particles coated with pseudoglobulin (symbol with solid center), and particles of denatured pseudoglobulin (solid symbol). Experiment at pH 3.0. Critical potential agglutination zone was between +13 to +15 millivolts and -13 to -15 millivolts. In this zone all particles flocculated out of suspension.

particles, but assume the cataphoretic charges and the critical potential agglutination level (14 millivolts) of the denatured protein. That is to say, they now behave, in these respects, as if they are particles of denatured pseudoglobulin.

⁵ The experiments were conducted at this pH because Loeb believed that coating occurred only on the acid side of the isoelectric point of the protein used. Dr. Northrop, in a personal communication, states that evidence has been obtained in his laboratory that coating occurs on the alkaline side as well.

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scribed later. It was observed that while S cells were encapsulated, the R forms no longer possessed capsules.

The nomenclature of S and R was first used by Arkwright (10) to describe biological variations in single strains of bacteria. His observations included the occurrence of two forms of colonies in old cultures of bacilli of the intestinal group. The S organisms grew in colonies with a smooth surface while the R strains presented colonies with a rough and irregular surface. The terms S and R have been universally adopted to describe similar changes in other species of bacteria. Observations on encapsulated organisms, however, fall mainly within the *Pneumococcus* group. With these organisms, it has been shown that accompanying the change from S to R, there is a loss of agglutinability by specific sera, loss of capsule, and attenuation of virulence (10-19).

Cultures of Friedländer's bacillus, also, have been separated by earlier workers (20-24) into S and R components, although such a designation was not in use at the time. The method of decapsulation as devised by Porges (25) must also be considered of the same order. More recently, Friel (13) and Hadley (26) have reported the occurrence of the two varieties in individual cultures of Friedländer's bacillus and have attributed to them the accepted characteristics of S and R forms.

In this study, the criteria for the conversion of S organisms to R forms have been: (1) loss of capsule and mucoid characteristics, (2) loss of agglutinability in type-specific sera, (3) attenuation of virulence, and (4) the development of colonies which present under the microscope a rough and irregular surface. In the case of R cells derived from Type C, loss of virulence was not an accompanying change since none of the Type C strains were virulent for mice.

The present report deals with the immunological reactions of encapsulated and capsule-free strains of Friedländer's bacilli and anti-S and anti-R sera, and the serological behavior of encapsulated bacilli after removal of capsules by the chemical method of Porges.

Methods.

Loss of Capsule Formation Induced by Growth in Vitro.—The non-encapsulated or "R" strains employed in this study were derived from pure S strains. An S strain representative of each of the three fixed types and one strain chosen from Group X were transplanted daily in broth to which had been added 10 per cent of homologous immune serum. Plates, streaked at the time of each transplant, were examined microscopically after 18-24 hours incubation, as recommended by Reimann (18). Within 6-10 transplants, plate cultures were obtained in which a

IMMUNOLOGICAL RELATIONSHIPS OF ENCAPSULATED AND CAPSULE-FREE STRAINS OF ENCAPSULATUS PNEUMONLÆ (FRIEDLÄNDER'S BACILLUS).

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In a preceding paper (1) evidence was presented that bacilli of the Friedländer group are separable into sharply defined and specific types. Of 30 strains employed in the study, three specific types and a heterogeneous group were demonstrated by agglutination, agglutinin adsorption, protection, thread and precipitin reactions. These types have been designated Type A, Type B, and Type C, while the remaining unclassified strains were placed in a tentative group, Group X. In the light of the present studies, the difficulties encountered in previous attempts to interpret the immunological reactions of Friedländer's bacillus appear to be due in large measure to the failure to distinguish in the cell the type-specific from the species-specific antigen; and the failure to recognize in immune serum the two distinct antibodies provoked by the respective antigens. The application of this concept to the Friedländer group was suggested by studies carried out in this laboratory on the immunological relationships of the cell constituents of *Pneumococcus* (2-7). Briefly, this concept involves two separable and distinct antigens—the one a soluble specific substance (now identified as a carbohydrate) which endows the organism with type specificity; the other, a protein substance which exhibits only the common and undifferentiated characters of the species.

Since the relation of the capsular material of Friedländer's bacillus to type specificity has been demonstrated (1, 8, 9), it seemed of importance to determine the immunological relationships of encapsulated and capsule-free strains of this organism. For this purpose, S and R varieties of the bacterial cell were obtained as will be de-

immunization was derived from the S strain later used for agglutination.

TABLE I.
Agglutination of Friedländer S Strains by Anti-R Sera.

Antigen encapsulated strain	Anti-R sera						Anti-S sera			
	Type A			Type B			A	B	C	X
	1:1	1:5	1:10	1:1	1:5	1:10	1:5	1:5	1:5	1:5
Type A.....	—	—	—	—	—	—	++++	—	—	—
" B.....	—	—	—	—	—	—	—	++++	—	—
" C.....	—	—	—	—	—	—	—	—	++++	—
Group X.....	—	—	—	—	—	—	—	—	—	++++

* The figures in this row represent ultimate dilution of serum.

++++ indicates compact disk agglutination with clear supernatant; —, no reaction.

TABLE II.

Precipitation of the Soluble Specific Substance of Friedländer's Bacillus by Anti-R Sera.

Antiserum	Soluble specific substance of Friedländer's bacillus									
	Type A					Type B				
	2	20	50	100	250	2	20	50	100	200
Type A (R).....	—	—	—	—	—	—	—	—	—	—
" B ".....	—	—	—	—	—	—	—	—	—	—
Group X ".....	—	—	—	—	—	—	—	—	—	—
Type A (S).....	—	+	++	+++	+	—	—	—	—	—
" B ".....	—	—	—	—	—	++	+++	++++	++++	++
Normal.....	—	—	—	—	—	—	—	—	—	—

* The dilutions are expressed in thousands.

++++ indicates heavy, compact, disk precipitate; +++, marked disk precipitate; ++, thin, film-like scale; +, ground glass turbidity.

Further evidence of the lack of type-specific antibodies in anti-R sera was sought by the precipitin reaction. Solutions of the isolated soluble specific substances of Types A and B were tested against three anti-R sera. As demonstrated in Table II, these sera contained no pre-

number of R colonies were observed. Single, typical R colonies were then transplanted into plain broth and the resultant R culture showed loss of capsule and attenuation of virulence. In each instance, the R culture failed to kill white mice, in doses of 0.5 cc., while the parent S strain from which the R had been derived regularly killed within 48 hours at a dilution of one ten-millionth cc.

Destruction of Capsule by Chemical Means.—The method devised by Porges was used. S strains were grown on agar slants and the organisms were washed off and suspended in salt solution. The suspensions were made acid with one-fourth volume N/4 HCl and heated at 80°C. for 15 minutes. The suspensions were then cooled immediately under tap water and neutralized with an equivalent quantity of N/4 NaOH. The exposure to heat was varied from 60°C. to 100°C. and from 10 minutes to 30 minutes without appreciable differences. No spontaneous clumping of the treated organisms was experienced when freshly prepared suspensions were used. After standing in the ice chest several days, however, preparations from two different strains became granular.

The method of immunization and the reaction of agglutination, precipitin and protection tests were conducted in the manner described in the preceding paper (1).

EXPERIMENTAL.

I. Immunological Reactions of "S" (Encapsulated) Strains in Immune Sera.

(a) *Anti-S Sera.*—It has already been shown in the preceding paper (1) that antisera prepared by immunization with encapsulated strains possess type-specific immune bodies, which agglutinate the encapsulated organisms and precipitate the soluble specific substance derived from them. In addition, such sera confer specific protection upon white mice against infection with strains of homologous types.

(b) *Anti-R Sera.*—Since the anti-R sera were prepared against strains of organisms which were capsule-free and which had lost the function of elaborating the type-specific soluble substance, it was to be anticipated that these sera would be lacking in type-specific antibodies. Definite proof of this, however, was obtained by determining the agglutination of S strains in anti-R sera. For purposes of comparison, the specific reactions of these same strains in anti-S sera of the homologous type were included. The results presented in Table I show conclusively that encapsulated strains are not agglutinated by sera prepared by immunization with the capsule-free (R) variants. This is true even though the organism employed for

ble protection against infection with an encapsulated (S) strain of Type A or Type B. Similarly, an anti-R serum prepared by immunization with a capsule-free organism derived from Type B offers no protection against infection with virulent strains of either of these specific types.

In summary, then, the foregoing experiments show that antisera prepared by the immunization of rabbits with degraded, capsule-free strains of Friedländer's bacillus (R forms) are devoid of specific agglutinins, precipitins, and protective antibodies for the virulent, type-specific, encapsulated bacilli. Anti-R sera, therefore, exhibit none of the type-specific reactions which characterize anti-S sera.

II. Immunological Reactions of "R" (Non-Encapsulated) Strains in Immune Sera.

(a) *Anti-S Sera.*—The anti-S sera used for the determination of specific types (1) were prepared in such manner as to avoid or at least minimize the concurrent presence of the common specific antibody. As was pointed out in the preceding paper, this may be accomplished most successfully by using young cultures of encapsulated organisms and by avoiding prolonged immunization. It was considered of interest to determine the agglutinative action of the type-specific sera against R strains of different origin. The results of these tests are given in Table V. It is seen that an immune serum prepared against an encapsulated strain of Type A contains only a small amount of the species antibody. In addition, this antibody is shown to be equally operative against four R strains, each of which in turn had been derived from an S organism of a serologically different type. The three anti-S sera of Type B, Type C, and Group X contained only minute and, for practical purposes, negligible traces of the R or common species antibody. Thus, further confirmation is advanced for the concept that the R organisms, devoid of capsules, are no longer type-specific.

(b) *Anti-R Sera.*—Evidence has been presented that capsule-free strains of Friedländer's bacillus are lacking in the ability to engender type-specific antibodies. However, they stimulate the formation of antibodies which react not only with the particular strain used for immunization, but with all other R forms regardless of the type from

capitins for the type-specific substances just as in the preceding experiment they were shown to possess no agglutinins for the encapsulated cells from which these substances were derived.

It has been shown in the previous communication that anti-S sera contain antibodies which afford specific protection in mice against

TABLE III.

Protection Offered by Anti-R Sera against Infection by Type A, Friedländer's Bacillus.

Type A encapsulated culture	Anti-R sera derived from				Virulence controls
	Type A		Type B		
	Amount	Result	Amount	Result	
"	"		"		
.001	.2	D. 16 hrs.	.2	D. 20 hrs.	
.0001	.2	" 19 "	.2	" 20 "	
.00001	.2	" 39 "	.2	" 22 "	D. 24 hrs.
.000001	.2	S.	.2	" 65 "	" 39 "
.0000001					" 65 "

S. indicates survival; D., death, the numerals representing the number of hours before death occurred.

TABLE IV.

Protection Offered by Anti-R Sera against Infection by Type B, Friedländer's Bacillus.

Type B encapsulated culture	Anti-R sera derived from				Virulence controls
	Type A		Type B		
	Amount	Result	Amount	Result	
cc.	cc.		cc.		
.001	.2	D. 15 hrs.	.2	D. 15 hrs.	
.0001	.2	" 15 "	.2	" 15 "	
.00001	.2	" 39 "	.2	" 24 "	D. 16 hrs.
.000001	.2	" 22 "	.2	" 65 "	" 39 "
.0000001					" 39 "

infection with virulent encapsulated bacilli of homologous types. Accordingly, an analysis of anti-R sera was made to determine the presence of protective properties. The results of these determinations are given in Tables III and IV. An anti-R serum prepared by immunization with a degraded Type A organism affords no measura-

TABLE VI.
Cross-Agglutinations of R Strains of Friedländer's Bacillus by Anti-R Sera.

Anti-R sera	Antigen derived from	Final dilution of serum										Normal serum 1:5
		1:5	1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560	
Type A	Type A	++	++	++	++	++	++	++	++	++	+	
	" B	++	++	++	++	++	+	++	++	++	-	
	" C	++	++	++	++	++	++	++	++	++	-	
	Group X	++	++	++	++	++	++	+	+	++	-	
" B	Type A	++	++	++	++	++	++	++	+	++	-	
	" B	++	++	++	++	++	++	++	++	++	+	
	" C	++	++	++	++	++	++	++	++	++	-	
	Group X	++	++	++	++	++	++	++	++	++	+	
Group X	Type A	++	++	++	++	++	++	++	++	+	-	
	" B	++	++	++	++	++	++	++	++	++	-	
	" C	++	++	++	++	++	++	++	++	++	-	
	Group X	++	++	++	++	++	++	++	++	++	+	

++++ indicates complete agglutination with flocculent sediment and clear supernatant; +++ almost complete, supernatant clouded; ++, marked agglutination; +, slight agglutination; -, no agglutination.

which they were derived. This fact is revealed in the cross-reactions of agglutination presented in Table VI in which all R strains are shown to be reciprocally agglutinated by each of the different anti-R sera.

The agglutination of the R strains in anti-R serum is characteristically different from the agglutination of S strains in anti-S serum of the homologous type. It will be recalled that with S strains, the reaction results in the formation of a compact, firm disk of agglutinated bacteria and occurs almost immediately in the more concentrated dilutions of the serum. The R organism, on the other hand, agglutinates very slowly, and in the higher concentrations of serum forms a fluffy precipitate; while in greater dilutions of the serum a fine, granular agglutination occurs which is difficult to read without a

TABLE V.

Agglutination of Friedländer R Strains by Anti-S Sera.

Antigen capsule-free strain	Anti-S sera											
	Type A			Type B			Type C			Group X		
	1:1	1:5	1:10	1:1	1:5	1:10	1:1	1:5	1:10	1:1	1:5	1:10
Type A.....	++	+	+	±	-	-	-	-	-	-	-	-
" B.....	++	+	+	±	-	-	-	-	-	-	-	-
" C.....	++	+	+	±	-	-	+	-	-	+	-	-
Group X.....	++	+	+	±	-	-	+	-	-	+	-	-

lens. The agglutinins in anti-R sera, which react with capsule-free strains, bear a marked similarity to the "fine flaking" agglutinins for the H form of *proteus* and typhoid bacilli described by Felix (27) and to the somatic agglutinins for the non-motile forms of hog-cholera bacillus reported by Orcutt (28). Furthermore, the agglutinin titre of anti-S sera is low (1:40-1:80) while the agglutinin titre of anti-R sera is high (1:2500).

Corroborative evidence of the serological identity of R strains was gained by the agglutinin adsorption test. Each anti-R serum was adsorbed with heat-killed suspensions of each R strain until all the agglutinins were removed for the adsorbing organisms. The adsorbed sera were then tested for the presence of agglutinins for other R strains. Repeated tests yielded constant results. To avoid un-

which are heated in the presence of acid are not agglutinated appreciably in purely anti-S sera of types which are serologically different from that yielding the decapsulated cells. Agglutination in such sera depends upon the presence of species antibody. In anti-sera of the parent strains, however, there is a definite precipitin reaction which is referable to the presence of unhydrolyzed soluble specific substance. In this connection it will be recalled (1, 9) that the capsular material of Friedländer's bacillus is precipitated by anti-S serum of the homologous type. Since the method of Porges strips the bacillus of the capsule with apparently only partial hydrolysis, it is not surprising that precipitation of the soluble specific substance is observed.

TABLE VIII.

Agglutination by Anti-S Sera of Suspensions of "S" Strains Decapsulated by Porges' Method.

Antigen derived from	Anti-S serum								
	Type B			Type C			Group X		
	1:1	1:5	1:10	1:1	1:5	1:10	1:1	1:5	1:10
Type A.....	+	—	—	+	+	—	+	+	—
" B.....	*++++	++	+	+	+	—	+	+	—
" C.....	+	+	—	*++++	++	+	++	+	—
Group X.....	+	+	—	+	+	—	*++++	++	+
Gran.....	+	+	—	+	±	—	++	+	—

* Typical "S" reaction.

(b) *Anti-R Sera*.—In anti-R sera, however, chemically decapsulated suspensions show a marked agglutination to a high dilution of serum and the reaction gives the characteristic appearance of R agglutination. The suspensions react equally well in all anti-R sera regardless of their type derivation. (Table IX.)

It is interesting to note that a closely related organism, *Encapsulatus granulomatis*, when subjected to the same conditions, is also agglutinated by anti-R sera. This explains in a measure the confusion experienced with allied encapsulated Gram-negative organisms. The species antigen of the Friedländer's bacillus is related to some extent to the species antigen of what have been considered allied organisms.

necessary repetition, a single typical protocol is given in Table VII. It is evident that the adsorption of an anti-R serum with any R strain removes from the serum agglutinins for the homologous organism as well as for heterologous strains. It is apparent, therefore, that immunologically all R cells are identical, as tested by the reactions of agglutination and agglutinin adsorption.

TABLE VII.

Agglutinin Adsorption.

Results of Agglutination with Anti-R Serum (Type B) after Adsorption by R Strains Derived from Homologous and Heterologous Types of Encapsulated Organisms.

Antigen capsule-free strain from	Anti-R serum (Type B) after adsorption with R strains derived from											
	Type A				Type B				Type C			
	20	50	100	500	20	50	100	500	20	50	100	500
Type A.....	—	—	—	—	—	—	—	—	—	—	—	—
" B.....	—	—	—	—	—	—	—	—	—	—	—	—
" C.....	—	—	—	—	—	—	—	—	—	—	—	—
Group X.....	—	—	—	—	—	—	—	—	—	—	—	—

* Figures represent ultimate dilution of serum.

III. Immunological Reactions of "S" (Encapsulated) Strains after Decapsulation by Porges' Method.

Appreciating the difficulties in the agglutination of Friedländer's bacillus, Porges devised a method of removing the capsule by weak acid in order to render strains more antigenic and better agglutinable. To test the effect of decapsulation on the serological behavior, suspensions of encapsulated bacilli representative of each of the specific types were subjected to the Porges technique. The cells were then tested for agglutinability in sera prepared by immunization with encapsulated and capsule-free organisms, respectively. For purposes of comparison, a strain of *Encapsulatus granulomatis* was included in the experiment.

(a) *Anti-S Sera.*—The results obtained with anti-S sera are summarized in Table VIII. It is evident that encapsulated organisms

provided immunization has not been intensive. The experience of former investigators, including our own, has been that in general prolonged immunization, even with capsule-bearing organisms, has yielded a serum so abundant in the species antibody as to obscure type-specific reactions. It is now recognized (7) in this connection that dissociation of the bacterial antigen occurring spontaneously *in vitro* and *in vivo* is a factor to be considered in immunization. That the dissociation observed in this study is due to a cleavage of the specific antigen complex in the animal body rather than a condition of cultural development is substantiated by certain evidence to be presented in a later paper.

"R" strains of Friedländer's bacillus on the other hand are characterized by loss of capsule formation, loss of the elaboration of soluble specific substance, and by attenuation of virulence. The sera of animals immunized to R strains contain only the species antibody. In other words anti-R sera agglutinate only capsule-free strains, irrespective of their type antecedent. Furthermore, anti-R sera fail to react with encapsulated organisms. They afford no passive protection against infections with virulent type-specific strains of Friedländer's bacilli, and they do not precipitate the soluble specific substance derived from organisms of homologous or heterologous type.

It is obvious, therefore, that the nature of antibody response in the animal is dependent upon the character of the bacterial cell which is utilized for immunization. Immunization with encapsulated strains engenders type-specific antibodies. On the other hand, a bacterial culture composed of both encapsulated and capsule-free cells, as is often the case, induces both the type-specific and species-specific antibodies. The predominance of the S or R component determines the predominance of the one or the other antibody. In either case confusing cross-agglutination reactions will be encountered, as they have been in the past, which are difficult of interpretation unless cognizance is made of the underlying principles. Immunization which is effected with cells devoid of capsules, as advocated by former workers (20, 21, 25), gives rise to only the species antibody which exhibits none of the type relationships. Similarly, in the agglutination reaction will be reflected the composition of a strain.

To recapitulate, encapsulated strains (Friedländer's bacillus) are transformed into "R" strains by heating in acid solution. The transformed cells react identically as "R" cells obtained by cultural methods. They agglutinate in anti-S sera depending upon the presence of species antibody, and in anti-R sera they behave as typical "R" strains.

TABLE IX.

Agglutination by Anti-R Sera of Suspensions of "S" Strains Decapsulated by Porges' Method.

Anti-R sera	Antigen derived from	Dilution of serum				
		1:50	1:100	1:250	1:500	1:1000
Type A	Type A	+++	+++	++++	++++	+++
	" B	++++	++++	++++	+++	++
	" C	+	++	+++	++	+
	Group X	+++	++++	++++	++++	+++
	*Gran.	++	++	+	—	—
" B	Type A	+++	+++	++++	++++	+++
	" B	++++	+++	++	+	—
	" C	++	++++	++	+	—
	Group X	++++	++++	++++	+++	++
	Gran.	+++	++	++	+	—
Group X	Type A	++++	++++	++++	++++	+++
	" B	++++	++++	++	++	+
	" C	++	+++	++++	+++	++
	Group X	++++	++++	++++	+++	++
	Gran.	+++	++	+	—	—

* This organism was isolated from an infection of granuloma inguinale.

DISCUSSION.

"S" strains of Friedländer's bacillus are characterized biologically by mucoid growth in liquid media, by capsule formation, and by exalted virulence. As antigens they give rise in the serum to type-specific antibodies. Consequently, such an immune serum will agglutinate all organisms of the homologous type, afford passive protection in white mice against infection by strains of the same type, and precipitate the soluble specific substance derived from the type strains. The extent of species antibody in anti-S sera is negligible

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The studies on the cell constituents of *Pneumococcus* to which reference has already been made reveal a striking similarity in the immunological behavior of the two encapsulated races. There has been observed in both species the occurrence of encapsulated, virulent organisms which differ serologically and which undergo degradation to such an extent as to become capsule-free, avirulent, and serologically undifferentiated.

These principles find a remarkable analogy even among flagellated bacteria as is indicated by Smith and Reagh (29), Orcutt, and Felix. These authors have shown independently that certain differences are demonstrable in the serological behavior between flagellated and non-flagellated organisms of the same strain. These differences have been related to the presence of two antigens, the flagellar or ectoplasmic, and the somatic or endoplasmic antigen.

CONCLUSIONS.

1. "S" strains of Friedländer's bacillus produce capsules, soluble specific substance, and are of exalted virulence. "S" strains are type-specific and react with only the type-specific antibodies of the homologous types.

2. Immunization with "S" cells induces the formation of antibodies which agglutinate type specifically, precipitate the corresponding soluble specific substance, and protect white mice against infection caused by organisms of the same type.

3. "R" strains of Friedländer's bacillus produce no capsules, produce no soluble specific substance, and are not pathogenic. "R" strains are serologically undifferentiated from each other and react with only the species antibodies.

4. Immunization with "R" cells induces antibodies which do not agglutinate encapsulated organisms, do not precipitate soluble specific substance, and do not afford protection against infection by Friedländer's bacillus. Anti-R serum contains only the species antibody which reacts with any capsule-free organism regardless of its type origin.

5. Decapsulation of "S" cells by heat and acid chemically converts a type-reacting organism into a species-reacting organism.

venous and intraperitoneal inoculation gave rise to a fatal systemic infection, which was not accompanied by local lesions, and which, except for the slight degree of anemia present, resembled a case of simple Oroya fever; the fever was severe (104–105°C.) and continuous, and the parasites were present in the blood in high titer and also in the lymph glands, spleen, and bone marrow. Monkey 7 was inoculated intradermally and intravenously with both passage virus and cultures, and the reaction was constitutionally as well as locally severe; *Bartonella bacilliformis* was isolated from blood diluted 1:10,000,000, from nodular tissue diluted 1:10,000, and also from the lymph glands, spleen, and bone marrow. Monkey 8, on the other hand, while receiving considerable amounts of passage virus both locally and intravenously, manifested no local and only mild systemic symptoms. Monkey 18 is of special interest as an illustration of a severe type of verruga such as may result from the inoculation of monkeys with human verruga tissues,^{2,3} while the striking fact brought out in the case of Monkey 25 is the simultaneous occurrence of severe symptoms of both verruga and Oroya fever, typical verruga nodules appearing spontaneously at sites remote from those of local inoculation. These appearances had not been observed by previous investigators in monkeys inoculated with human verruga tissues.

Quantitative estimates of the number of *Bartonella bacilliformis* present at any given time in the blood are expressed in terms of the highest dilution of the blood from which cultures of the organism could be obtained. The culture method is the only satisfactory test of the presence of the parasite. The use of monkeys is obviously not practicable for quantitative determination and would not, moreover, be reliable because of the variation in susceptibility to *Bartonella* infection. Careful microscopical examination of stained blood films was not, of course, neglected, but when one considers that citrated blood of as high titer as 1:100,000 would show only 1 cell invaded by the parasite among 3,000 cells (1 cc. of citrated blood containing approximately 3 billion red cells), the difficulties of this method of demonstrating the organism become evident.

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ETIOLOGY OF OROYA FEVER.

III. THE BEHAVIOR OF *BARTONELLA BACILLIFORMIS* IN *MACACUS RHESUS*.

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PLATES 22 to 24.

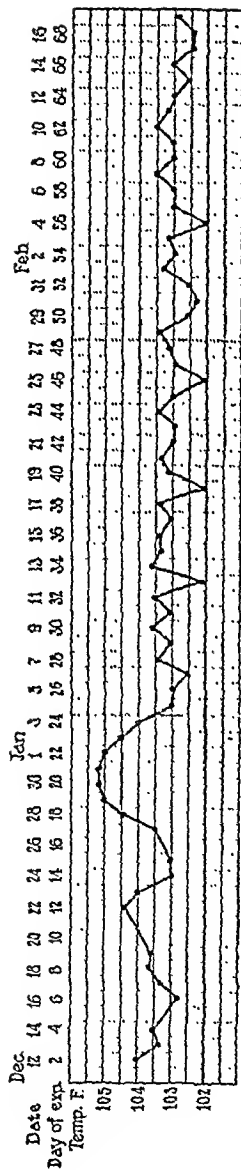
(Received for publication, June 12, 1926.)

As reported in a previous paper,¹ a strain of *Bartonella bacilliformis* isolated from the blood of a fatal case of Oroya fever when inoculated into young *Macacus rhesus* monkeys induced a protracted course of intermittent fever, resembling that observed in some human cases of Oroya fever. The microorganism was demonstrated in the red cells of these animals and recovered in culture, but the clinical manifestations in monkeys differed from those of the human disease in that none of the animals showed the severe anemia so characteristic of Oroya fever. It was also shown that intradermal inoculation of the cultures into the shaved skin of the eyebrows of the monkeys gave rise to a granulomatous nodule rich in capillaries, in which the microorganisms could be demonstrated by section and by culture. The nodules presented a marked resemblance to those of verruga peruviana.

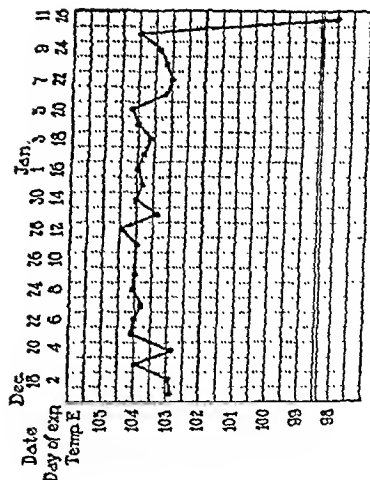
Further study of the behavior of *Bartonella bacilliformis* in *rhesus* monkeys, particularly with reference to the effects of passage strains, has brought to light a number of additional facts, which throw some light on the variety of manifestations of *Bartonella* infection in man.

The protocols are presented in chronological order, and the extraordinary variability in response to inoculation of the parasite is illustrated by all of them. The severity of infection in most instances is referable probably to enhanced virulence of the parasite through adaptation to the animal. In Monkey 5 we have an example of severe systemic infection induced by local inoculation. In Monkey 6 intra-

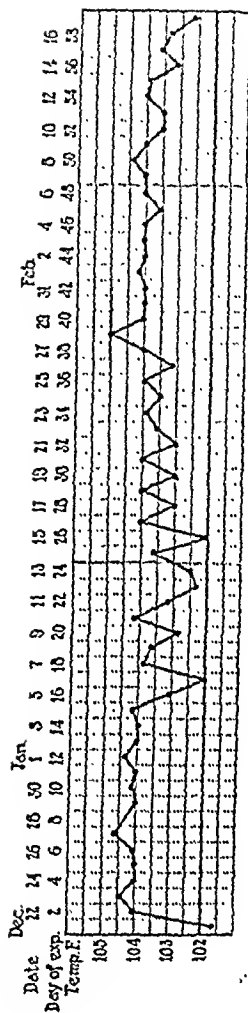
¹ Noguchi, H., and Battistini, T. S., *J. Exp. Med.*, 1926, **xlili**, 851.



TEXT-FIG. 1.



TEXT-FIG. 2.



TEXT-FIG. 3.

The Invasion of the Blood by B. bacilliformis after Intradermal Inoculation.

The ease with which *Bartonella bacilliformis* may enter the general circulation after intradermal inoculation is illustrated in the case of *M. rhesus* 5.

M. rhesus 5 (Text-fig. 1). A young monkey, about 2,000 gm.—a size which has been found most satisfactory for experimental work with *Bartonella bacilliformis*—was inoculated, Dec. 11, 1925, intradermally on the shaved left eyebrow, with 0.2 cc. of a saline suspension of pooled cultures which on dark-field examination showed 3 to 4 motile microorganisms per field. The mixture included 1 cc. of condensation water from a 9 day old culture on a blood agar slant, derived from the original human blood, and 0.3 cc., in 20 cc. of 0.9 per cent saline, of a 10 day old culture, derived from the blood of *M. rhesus* 1, and grown on leptospira medium at 25°C. Cultures made with the blood withdrawn on that date (11 days after inoculation) revealed the presence of *Bartonella bacilliformis* in a titer of 1:100,000, and in stained preparations the parasites were demonstrated in small numbers in the erythrocytes. Cultures made on Dec. 28, 1925, and on Jan. 5, 12, and 25, 1926, also yielded positive results. The animal showed the highest fever during this period, the temperature being continuously at 105°F. from Dec. 29 to Jan. 1. The last two specimens of blood had a titer of only 1:10, and the parasites could no longer be demonstrated in stained smears, hence the number of organisms in the blood stream had diminished rapidly within a period of 24 days. The site of intradermal inoculation on the left eyebrow, however, began to show induration nearly 42 days after injection; on Jan. 22 it was about 2 × 3 mm. in area and was slightly raised; on Feb. 1 it measured 6 × 6 mm., and on Feb. 4 about 9 × 9 mm. It was at first pale and semitransparent, but firm to the touch; then it became a deep rose-red and finally cherry-like in form and color. During the following 10 to 12 days it remained nearly stationary, but later it showed slight excoriation at the apex and was removed* for transfer, culture, and histological studies. It proved to be infective for *rhesus* monkeys, yielded a pure culture of *Bartonella bacilliformis*, and on section showed the characteristic endothelial proliferation with intracellular localization of the organism.

Blood cultures made on Feb. 9 and 17 yielded growth of *Bartonella bacilliformis* in 0.1 cc. of a 1:10 dilution. At no time after subsidence of the early violent febrile reaction had the animal's temperature been higher than 103.6°F. The animal was killed under ether anesthesia on Feb. 17, 1926, 63 days after inoculation, in order that the distribution of the microorganisms and the character of the specific lesions might be ascertained.

* All operations were performed under ether anesthesia.

Histologically the nodules were the products of infection solely with *Bartonella bacilliformis* and they showed the characteristic localization of the organism in the endothelial cells of the lesions. An interesting difference in the number of organisms present in the nodule and the scarified area (both induced by the emulsion of nodule from Monkey 4) was revealed by titration. The emulsion of the skin lesion yielded growth in a 1:10,000 dilution, while that of the nodule contained sufficient numbers of organisms in a 1:10 dilution to yield a culture, but not in a 1:100 dilution. It may be that the age of the lesion is a factor in this quantitative difference, since the more slowly progressing lesion contained the larger number of organisms.

Blood counts by Dr. Bauer gave the following results:

Jan. 4, 1926 (14 days after inoculation).	Erythrocytes 4,400,000.	
" 12, " (22 " " ").	" 4,620,000.	Hemo-
		globin 75 per cent.

Feb. 17, 1926 (58 days after inoculation).	Erythrocytes 5,472,000.	Hemo-
		globin 80 per cent.

It is difficult to draw any conclusion from the fragmentary blood counts. The animal appeared, however, to be in good condition when it was sacrificed on Feb. 17 for examination of various organs (58 days after inoculation).

Autopsy.—The lungs showed a few scattered grayish nodules, the liver a few small grayish spots. The spleen was enlarged and granular; the lymph nodes moderately swollen. The kidneys were normal. *Bartonella bacilliformis* was obtained in culture from the spleen, bone marrow, lymph glands, and heart blood.

In the foregoing experiment we were apparently dealing with a protracted and severe but non-fatal, *Bartonella* infection, with possible visceral involvement. It is of special interest that the local and systemic processes proceeded simultaneously.

That an unusually resistant animal may occasionally be encountered among *Macacus rhesus* monkeys is shown by the following protocol.

M. rhesus 8 was inoculated on Dec. 30, 1925, with an emulsion of the well developed nodule from *M. rhesus* 4.¹ The material was introduced intradermally, by scarification on both eyebrows, and also intravenously (2 cc.). During 50 days of observation there were, roughly speaking, three periods of high fever (Text-

Autopsy.—Spleen enlarged and granular on surface, culture +. Lymph glands everywhere enlarged, culture +. Heart blood culture +. Bone marrow (femur) reddish, culture +. Lungs normal, culture negative. Liver normal, culture negative. Kidney normal, culture lost because of contamination.

With the cooperation of Dr. J. H. Bauer, erythrocyte counts and hemoglobin estimates⁴ were made on several occasions. The results follow:

Jan. 12, 1926 (32 days after inoculation). Erythrocytes 6,169,000. Hemoglobin 82 per cent.

Jan. 25, 1926 (45 days after inoculation). Erythrocytes 5,901,000. Hemoglobin 75 per cent.

Feb. 15, 1926 (66 days after inoculation). Erythrocytes 5,248,000. Hemoglobin 70 per cent.

Feb. 17, 1926 (68 days after inoculation). Erythrocytes 4,856,000. Hemoglobin 70 per cent.

The chronic nature of the infection is clearly indicated by the persistence of the parasites in the peripheral blood, bone marrow, lymph nodes, and spleen. The number of microorganisms demonstrated by stained preparations in the blood corpuscles was never large at any time, even during the febrile or "septicemic" period. There was little sign of anemia until the latter part of the experiment, when the number of red cells seemed definitely to have diminished, notwithstanding the marked fall in temperature and the scarcity of *Bartonella bacilliformis* in the blood at this time.

Variability in the Effect of Inoculation of Bartonella bacilliformis into Macacus rhesus.

The outcome of inoculation of a given pathogenic microorganism must depend in part on the virulence of the strain and in part on the degree of susceptibility of the host. In the case of a newly isolated microorganism the effect of successive animal passages on virulence is problematic; the pathogenic property may be either weakened or enhanced by continued passage. Experiments have just been described in which a second passage of *B. bacilliformis* through *Macacus rhesus* indicated a decided increase in virulence of the organism for this species of animal. The present experiment has to do with the effects of further direct passages in *rhesus* monkeys.

⁴Dr. J. H. Bauer was kind enough to make the blood counts on all the animals of this report. The hemoglobin estimates were made by means of the Sahli hemoglobinometer.

of organisms were found in sections. Loss of virulence may have resulted from the final etherization of Monkey 4.

The comparatively mild infection, accompanied by severe febrile reactions, seems to have been due to a higher resistance of this particular animal, but the chronic nature of the *Bartonella* infection is indicated even in this instance by the persistence of the microorganisms in the lymph glands.

The Reproduction of Striking Clinical Features of Oroya Fever and Verruga.

Up to this point in the work, the manifestations by monkeys of infection with *Bartonella bacilliformis* resembled human Oroya fever or verruga in essentials, *i.e.*, in the typical localization of the organism and in the characteristic skin lesions, respectively, but the severe anemia of Oroya fever and the general eruption of verruga had not as yet been reproduced in monkeys.

In Monkey 18, however, the local lesions, while not general or spontaneous, attained extraordinary size and resembled those described by previous investigators as resulting from the inoculation of suspensions of human verruga tissues into monkeys. Monkey 25 developed the spontaneous generalized eruption characteristic of verruga and also severe symptoms of Oroya fever (anemia, presence of *Bartonella bacilliformis* in considerable numbers in the erythrocytes). Although the period of fever was very brief in this animal, the manifestations of infection were similar to those of certain human cases in which Oroya fever and verruga are simultaneously present.⁵

M. rhesus 18 (Text-fig. 5) was inoculated on Feb. 15, 1926, with passage virus (suspension of nodular tissue) from Monkey 5. The shaved left eyebrow received two intradermal injections of the emulsion, and the shaved right eyebrow was scarified and smeared with the same material. The skin of the abdomen was shaved, and on the left side two intradermal injections were made of the emulsion, while on the right two scarified areas (about 3 × 3 cm. each) were spread with the same material. Induration at the sites of inoculation became evident in about 14 days and were definite after 26 days (Fig. 1). The lesions steadily increased in size, and 36 days after inoculation the largest of the nodules on the scarified areas of the right eyebrow measured 2.5 × 2 cm. and was 1 cm. high

⁵ Odriozola, E., La maladie de Carrion, Paris, 1896.

fig. 4). During the first 12 days the temperature rose as high as 106°F ., and during the second and third periods it fluctuated about 104° for about 10 days. Although the temperature was 104° on the day following inoculation, the febrile reaction seems to have become definite on the 6th day.

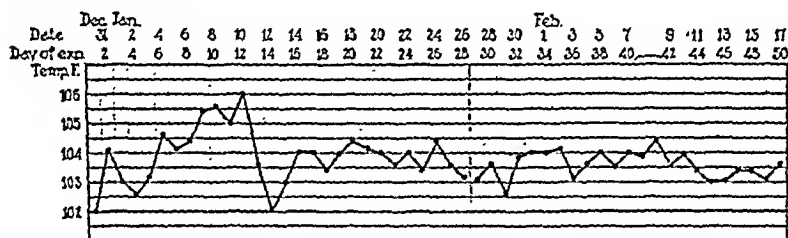
Four blood cultures were made during life and one post mortem, with the following results:

Jan. 4, 1926 (5 days after inoculation). Positive with blood diluted 1:10.
 " 8, " (9 " " "). " " undiluted blood.
 " 18, " (19 " " "). " " blood diluted 1:1,000.
 Feb. 3, " (35 " " "). " " " " 1:10.
 " 17, " (49 " " "). Negative.

Blood counts showed no anemia:

Jan. 8, 1926 (9 days after inoculation). Erythrocytes 5,506,000. Hemo-
 globin 80 per cent.

Feb. 3, 1926 (35 days after inoculation). Erythrocytes 5,414,000. Hemo-
 globin 80 per cent.



TEXT-FIG. 4.

Feb. 17, 1926 (49 days after inoculation). Erythrocytes 5,496,000. Hemo-
 globin 80 per cent.

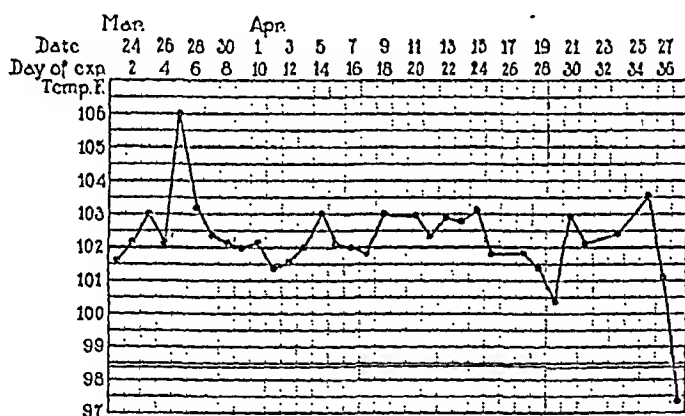
The animal was killed by etherization on Feb. 17, 1926, for examination. Autopsy revealed marked enlargement of the lymph glands. No other changes were noticeable. The spleen, blood, and bone marrow failed to yield cultures, but growth was obtained from the emulsion of the lymph nodes.

As a result of the inoculations on the eyebrows, four tiny indurated areas appeared within 9 to 10 days but did not enlarge and within 18 days had practically disappeared. A red spot of pin-point size was noticed on the scarified area after 29 days, but it also disappeared in another week.

While the nodule employed for inoculation in this instance probably contained fewer organisms than the one removed from Monkey 4 earlier in its evolution, yet the suspension from it yielded a pure growth of *Barlonella bacilliformis* in a 1:10 dilution, and a large number

One of the most striking features of the experimental *Barlonella* lesions is the slow continuous formation of granulomatous tissue on a scarified surface of the skin, which results in an enormous accumulation of endothelial cells and subsequent capillary formation, a process apparently identical with that which takes place in the lesions of verruga in man.

M. rhesus 25 (Text-fig. 6) was inoculated intravenously on Mar. 23, 1926, with 6 cc. of a saline suspension of cultures grown on blood slants for 10 days at 25°C. At the same time the shaved right eyebrow was inoculated intradermally as well as by scarification with a suspension of nodular tissue from *M. rhesus* 18. The left eyebrow was similarly inoculated with culture. This animal had

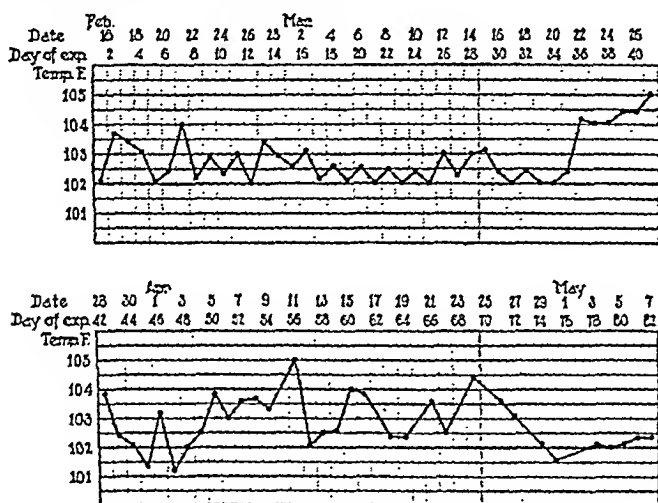


TEXT-FIG. 6.

only 1 day of high temperature, Mar. 27, when 106°F. was recorded; otherwise the course of disease was practically afebrile.

The first sign of infection in this instance was a peculiar condition of the upper eyelids, the edges of which were irregularly thickened, and perhaps a little pinkish on Apr. 7, 15 days after inoculation. Within a week numerous miliary, cherry-red nodules appeared around the eyes on both sides, and distinct nodules became noticeable at the site of intradermal inoculation as well as on the scarified areas. The animal presented a striking aspect (Fig. 4). These lesions, and several miliary nodules scattered over the body, especially near the right inguinal surface (Fig. 5) gradually became larger within the next 5 or 6 days and partly confluent. On the posterior surface of the left leg nodules of varying size appeared within 3 weeks after inoculation (Fig. 6) and remained until death. The eyes were virtually closed 36 days after inoculation (Apr. 28, 1926), when the animal died (Fig. 12). One of the spontaneous nodules was removed for cultural

(Fig. 7), while the two round subcutaneous nodules on the left abdominal wall stood out about 1 cm. and showed a reddish spot at the point of insertion of the syringe needle (Fig. 8). The lesions were of maximum size 59 days after inoculation (Fig. 2) and remained stationary for about 14 days. Retrogression was slow, but at the time of writing (99 days after inoculation) they are pale, fibrous, and considerably decreased in size, as shown in Figs. 3 and 11. No ulceration or softening of the lesions could be detected at any time. The examination of a nodule excised from the right eyebrow 26 days after inoculation revealed the characteristic granulomatous structure with proliferation of endothelial cells (Fig. 9), in the cytoplasm of which were found varying numbers of *Bartonella bacilliformis*, usually in clumps (Fig. 10).



TEXT-FIG. 5.

Bartonella bacilliformis was demonstrated in sections of the lesions and isolated in culture from emulsions of the tissue. It was recovered from the blood of the monkey on the following occasions:

Mar. 5, 1926 (18 days after inoculation). Positive in blood diluted 1:100,000.
 " 18, " (31 " " " " " " " " 1:100,000.
 " 27, " (40 " " " " " " " " 1:10.

Comparison of blood counts made on the day of inoculation and 60 days later show that there was a definite decrease in the number of red cells during the course of illness:

Feb. 15, 1926. Erythrocytes 5,356,000. Hemoglobin 90 per cent.
 Apr. 16, " " 4,120,000. " 60 " "

of infective material from the ulcerated eyelid to a needle wound caused by heart puncture. Notwithstanding this late stage invasion, the essential features of the course of illness in this animal were, I believe, the result of local and constitutional infection with *Bartonella bacilliformis*.

SUMMARY.

The experiments reported here were carried on in the main with passage strains of *Bartonella bacilliformis*, and the results indicate that the virulence of the organism has been considerably enhanced by passage through susceptible animals. While the animals of the earlier experimental series showed no anemia, some of the present group manifested a definite reduction in the number of red cells and in hemoglobin, and in one instance (*M. rhesus* 25) anemia was of the extreme type so often associated with Oroya fever in man. The anemic condition appeared to be secondary in character, however, nucleated red cells being few in number. In this animal also *Bartonella bacilliformis* was readily demonstrated in the erythrocytes by means of stained smears, though the number of cells invaded by the parasites was by no means so great as in the human infection.

In most instances of experimental *Bartonella* infection so far induced the demonstration of the parasites by ordinary routine examination of stained film preparations is possible only when the titer of the blood exceeds 1:1,000. Prolonged search of many slides has not been attempted, however. The number of microorganisms in the blood, as shown by culture tests of ascending dilutions, was in most instances highest (1:100,000 to 1:10,000,000) during the early period of the infection coincident usually with the period of highest fever, falling to a titer of 1:10 during the last half of the disease. In one of the fatally infected monkeys, however, the titer increased from 1:10 on the 4th day to 1:1,000,000 on the 24th day. The titer of the blood was equally great in Monkeys 5 and 6, although the former was inoculated locally, the other intravenously and intraperitoneally. The largest proportion of infected red cells was found in Monkey 25, while the blood titer, as shown by culture test, was highest in Monkey 7.

The febrile reaction varied in the animals of this series from a severe continuous fever of 104–105°F., lasting 2 to 3 months, in one instance,

and histological examination on Apr. 15. It showed the usual endothelial proliferation (Fig. 13), and large numbers of *Bartonella bacilliformis* were present (Fig. 14).

The number of red corpuscles infected with *Bartonella bacilliformis* was larger than in any of the monkeys previously studied, cells invaded by the parasites being readily demonstrable in film preparations. Curiously enough, however, the detection of the organism was not accomplished, even by the culture method, during the 48 hours preceding death.

Mar. 27 (5 days after inoculation). Positive with blood diluted 1:1,000.

Apr. 8 (18 " " "). " " " " 1:100,000.

" 16 (26 " " "). " " " " 1:1,000,000.

The results of blood counts by Dr. Bauer were as follows:

Mar. 27, 1926 (4 days after inoculation). Erythrocytes 5,288,000. Hemoglobin 85 per cent.

Apr. 20, 1926 (28 days after inoculation). Erythrocytes 3,240,000. Hemoglobin 45 per cent.

Apr. 22, 1926 (30 days after inoculation). Erythrocytes 2,992,000. Hemoglobin 40 per cent.

Apr. 24, 1926 (32 days after inoculation). Erythrocytes 3,368,000. Hemoglobin 40 per cent.

Apr. 26, 1926 (34 days after inoculation). Erythrocytes 2,736,000. Hemoglobin 35 per cent.

Apr. 27, 1926 (35 days after inoculation). Erythrocytes 2,120,000. Hemoglobin 30 per cent.

Apr. 28, 1926 (36 days after inoculation). Erythrocytes 1,624,000. Hemoglobin 25 per cent.

Autopsy.—Very much emaciated, wax-yellow. The nodules on the eyebrows remained unopened, but the lesions on the lids had partly ulcerated, and the tissues had become somewhat necrotic. The eyes were almost closed. There was some seropurulent discharge from the ulcerated nodules, which were nearly confluent. Corneæ not affected. Spontaneous miliary nodules were found on the abdomen, thighs, and legs, the posterior surfaces of both legs, in particular, being covered with cutaneous or subcutaneous nodules of varying size (Fig. 6), some adherent to the fascia. Some nodules were hemorrhagic.

There was exudative pericarditis, due to the presence of minute Gram-negative, motile bacilli in enormous numbers, especially within the polymorphonuclear leucocytes. The heart muscles were pale and flabby, but the pleuræ did not appear to be involved. Lungs normal. Liver perhaps enlarged, and pale. Spleen somewhat enlarged and soft. Stomach empty, normal. Intestines and mesentery: General lymphatic system hypertrophied everywhere. Other organs normal. Bone marrow (femur) dark red.

The findings described suggest a terminal secondary bacterial pericarditis, which may have been the result of transfer by the animal

characteristic phenomena of both conditions are simultaneously present. Whether the appearance will resemble those of the one or the other condition appears to depend on the susceptibility of the individual as well as on the virulence of the organism. Moreover, it seems probable that different degrees of resistance to the invasion of the parasite on the part of the blood cells, internal organs, or skin of a given animal may determine the predominant clinical manifestations of the infection. The factor of variation in susceptibility of different individuals or different tissues of the same individual would account for the variety of types of human *Bartonella* infection.⁶

EXPLANATION OF PLATES.

PLATE 22.

All figures natural size.

FIG. 1. *M. rhesus* 18, 26 days after inoculation. The nodule on the inner aspect of the left eyebrow was subsequently removed for examination.

FIG. 2. *M. rhesus* 18, 59 days after inoculation, when the nodules had reached their maximum development. The nodules on the right side had been induced by inoculation by scarification; there was no softening or ulceration of these lesions at any time. The nodule on the left side was induced by intradermic inoculation.

FIG. 3. *M. rhesus* 18, 99 days after inoculation, when the nodules had retrogressed considerably and the animal was decidedly paler.

FIG. 4. *M. rhesus* 25, 23 days after inoculation. The indurations which had developed at the sites of inoculation on the inner aspect of each eyebrow were almost eclipsed by the numerous spontaneous deep red nodules which had appeared about the eyes.

FIG. 5. *M. rhesus* 25, showing the spontaneous nodules which developed in the inguinal region, 29 days after inoculation.

FIG. 6. *M. rhesus* 25, showing the spontaneous nodules which had appeared on the posterior surface of the legs 29 days after inoculation.

PLATE 23.

FIG. 7. *M. rhesus* 18, 36 days after inoculation, showing the appearance of the lesions at the sites of intradermal inoculation and of inoculation by scarification. Natural size.

FIG. 8. *M. rhesus* 18, 36 days after inoculation, showing the lesions on the abdomen. The verrucous lesions on the right side were induced by scarification,

⁶ Arce, J., La enfermedad de Carrion, Lima, 1920.

with a remittance during the 3rd to 5th weeks, to the acute high fever (106°F.) of 1 day's duration in the fatally infected monkey, No. 25. The more usual reaction, however, is an irregular course of moderate fever with one or more periods of high temperature (105°).

Bartonella bacilliformis was constantly demonstrated, both microscopically and by culture tests, in the lymph glands of animals sacrificed 2 to 3 months after inoculation, and in two of three instances it was present also in the spleen, bone marrow, and heart blood. In the case of *M. rhesus* 6, which died 26 days after inoculation, the microorganism was obtained also in culture from the lymph glands, spleen, and heart blood taken at autopsy. In the other animal which died, a terminal bacterial infection, while not obscuring the effects of the *Bartonella* infection, made it impossible to isolate the parasite from either blood or tissues.

The skin lesions, whether of the nodular type, induced by introduction of the virus intradermally or by application to the scarified skin, or of the miliary character occurring spontaneously as a result of systemic infection, always yielded cultures of *Bartonella bacilliformis*, and stained sections of such lesions revealed the parasites in large numbers in their characteristic situation in the endothelial cells.

A chronic, systemic infection, in which the lymph glands are enlarged and *Bartonella bacilliformis* is present in the blood in high titer, may be induced by local inoculation, as shown in the case of *M. rhesus* 5.

The local lesions induced in one instance by introduction of a passage strain, both intradermally and by scarification, attained within 2 months extraordinary size, the nodules arising at adjacent sites of inoculation on the right eyebrow having coalesced into a large pedunculated mass which overhung the eye. This type of reaction had not been observed hitherto in the course of the present study but has been described by earlier investigators as a result of the inoculation of monkeys with human verruga tissues.

The striking fact brought out in the present study is the variety of responses to inoculation which animals of the same species may manifest. The clinical features of the infection may be typical of Oroya fever or may resemble those of verruga peruviana, and in *M. rhesus* 25 we have an instance of a type of infection in which the

the two round subcutaneous nodules on the left side by intradermal and partly subcutaneous inoculation. Natural size.

FIG. 9. Section of nodule removed from eyebrow of *M. rhesus* 18, 26 days after inoculation. Giemsa's stain. $\times 182$.

FIG. 10. The same section at a magnification of 1,000 times, showing the presence of *Bartonella bacilliformis*.

FIG. 11. *M. rhesus* 18, 99 days after inoculation, showing the appearance of the receding lesions on the abdomen. One of the nodules at the sites of intradermal inoculation had been removed.

PLATE 24.

FIG. 12. *M. rhesus* 25, at the time of death 36 days after inoculation.

FIG. 13. Section of spontaneous nodule of *M. rhesus* 25, removed 23 days after inoculation. Giemsa stain. $\times 182$.

FIG. 14. The same section at a magnification of 1,000 times, showing the presence of masses of *Bartonella bacilliformis*.



FIG. 1. *M. rhesus* 18,
26 days after inoculation.



FIG. 4. *M. rhesus* 25,
23 days after inoculation.

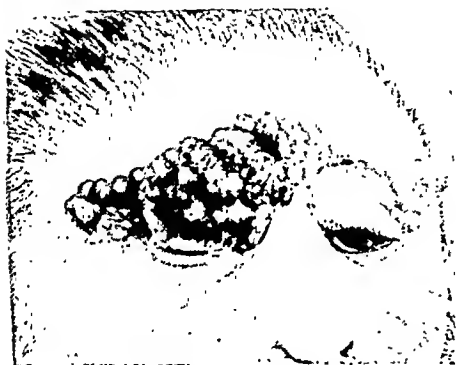


FIG. 2. *M. rhesus* 18,
59 days after inoculation.



FIG. 5. *M. rhesus* 25
(groin), 29 days after inoculation.

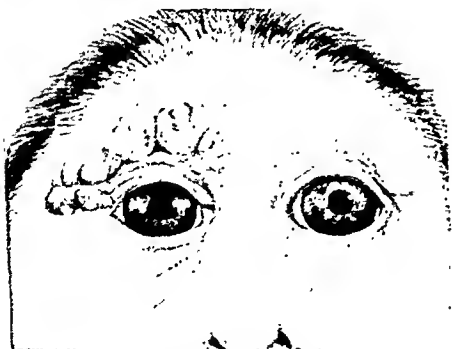
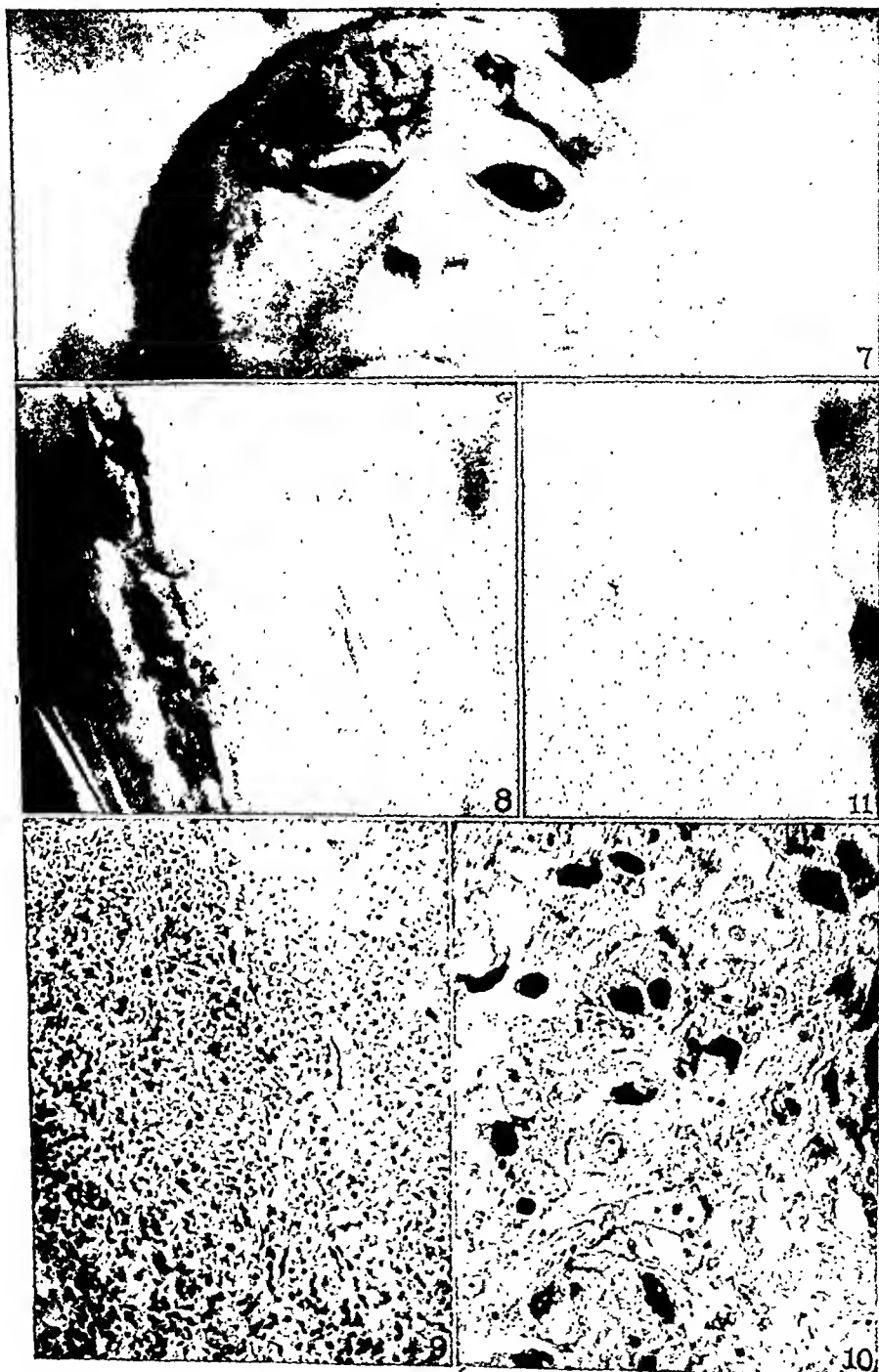


FIG. 3. *M. rhesus* 18,
99 days after inoculation.



FIG. 6. *M. rhesus* 25
(leg), 29 days after inoculation.





(Noguchi: Etiology of Oroya fever. III.)

was desirable to reproduce, if possible, the complete clinical picture of Oroya fever,—the extreme anemia of pernicious type, and the invasion of large numbers of the erythrocytes by the microorganism, such as takes place in human Oroya fever (Fig. 2). The opportunity presented itself of studying the effect of the inoculation of two anthropoid apes with cultures and passage strains of *Bartonella bacilliformis*, and the experiments were undertaken in the hope that these animals, because of their close phylogenetic relationship to man,^{3,4} might prove sufficiently susceptible to the infection to manifest all of the severe symptoms to which the parasite gives rise in man, either of the type of Oroya fever, or that of verruga. As the protocols show, however, they manifested only slight constitutional reaction to the inoculations. The erythrocytes were invaded by the organisms to a small extent only, and there was little or no anemia.⁵ The skin lesions, while of the characteristic verruga type, remained localized at or near the sites of introduction of the microorganisms; there was no spontaneous generalized skin eruption such as is observed in human verruga, and in rare instances in *rhesus* monkeys.

A young female chimpanzee (*Pan leucoprymnus*), said to be about 4 years old, was inoculated on Jan. 29, 1926, with the suspension of a nodule excised* from the eyebrow of *M. rhesus* 3¹ on the same day, and also with cultures, grown on leptospira medium, representing the second generation from the original human blood and the first generation from the blood of *M. rhesus* 1.¹ The suspension was inoculated intradermally into the right eyebrow and by scarification on the right side of the abdomen, while on the left eyebrow and left side of the abdomen a mixture of the suspension with cultures was introduced part intradermally and part subcutaneously. The quantities injected were 0.2 to 0.3 cc. 5 cc. of the mixture were injected subcutaneously into the right lower portion of the abdomen, above the inguinal region, the site of inoculation being carefully massaged after the injection.

The normal temperature of the chimpanzee, as shown by daily records covering a period of several weeks, varied between 98.6°F. and 99.6°; occasionally the afternoon (4 p.m.) temperature was as high as 100°. Blood counts made before inoculation on Jan. 29 gave the following results: Erythrocytes 5,520,000, leuco-

³ Nuttall, G. H. F., Blood immunity and blood relationship, Cambridge, 1904.

⁴ Landsteiner, K., and Miller, C. P., *J. Exp. Med.*, 1925, xlii, 853.

⁵ The hematological studies were by Dr. J. H. Bauer.

* All operations were performed under ether anesthesia.

ETIOLOGY OF OROYA FEVER.

IV. THE EFFECT OF INOCULATION OF ANTHROPOID APES WITH BARTONELLA BACILLIFORMIS.

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PLATES 25 TO 28.

(Received for publication, June 18, 1926.)

It has been reported in previous papers that the intravenous inoculation of young *rhesus* monkeys with a strain of *Bartonella bacilliformis* isolated from a case of Oroya fever gives rise to two essential manifestations of Oroya fever, namely, protracted fever, and typical localization of the parasite within the red blood cells (Fig. 1), while the introduction of the organism intradermally induces skin lesions which are indistinguishable from those of *verruca peruviana*.¹ It has been shown also that after intradermal inoculation the organism may pass into the circulation and invade the red blood cells, that it may give rise to an anemic condition comparable with that of Oroya fever, except for the absence of appreciable numbers of nucleated red cells in the blood stream, and that it may induce a generalized eruption similar to that of human *verruca*.² In most instances, however, the inoculated monkeys have shown little or no anemia, and comparatively few erythrocytes are invaded by the parasite, while the *verruca* nodules have usually arisen only at the sites of inoculation into the skin.

Since the invasion of the red corpuscles by the bacilliform organisms is the most characteristic sign of Oroya fever, and the formation of nodules by the proliferation of endothelial cells is equally typical of *verruca*, the findings described indicated that the organism cultivated was probably the cause of both these conditions. Nevertheless, it

¹ Noguchi, H., and Battistini, T. S., *J. Exp. Med.*, 1925, xliii, 851.

² Noguchi, H., *J. Exp. Med.*, 1926, xlv, 697.

cytes 7,810, hemoglobin 80 per cent.⁶ The inoculations were made between 12 noon and 1 p.m.

Jan. 30. Animal appeared as usual; appetite good. Slight irritation at the sites of scarification. Jan. 31. No change, except that the lines of scarification were slightly raised. Feb. 3. Some swelling at the sites of intradermal injection, but no reddening. Feb. 4. Animal appeared perfectly well. Erythrocytes 5,680,000, leucocytes 8,190, hemoglobin 85 per cent.

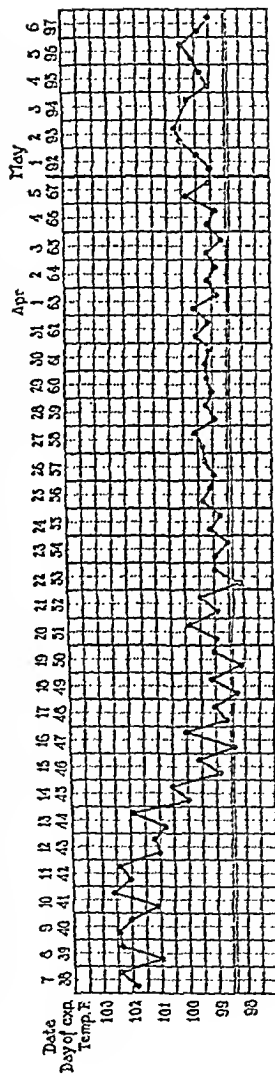
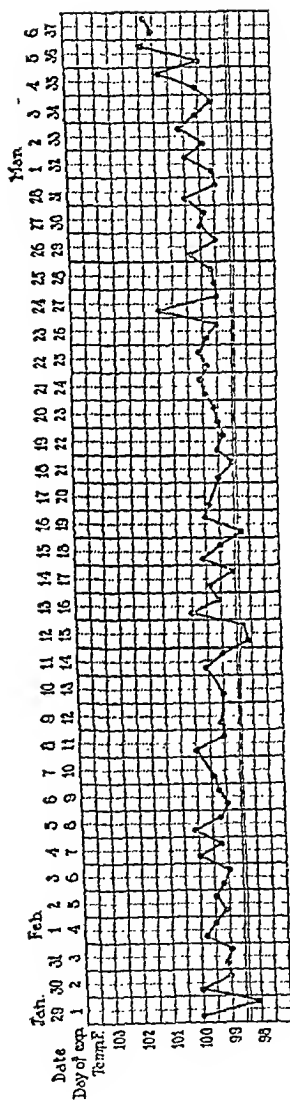
Feb. 8. The abdominal wall showed definite edema, but the animal ate well and appeared normal. Erythrocytes 5,335,000, leucocytes 11,900, hemoglobin 85 per cent. The blood yielded cultures of *Bartonella bacilliformis* in a dilution of 1:10, but routine examination of film preparations failed to demonstrate cells containing the organism. Feb. 9. Distinct induration at the sites of intradermal inoculation on eyebrows and abdomen, but no reddening. Feb. 12. Abdominal nodule at site of intradermal and subcutaneous injection increased in size (3×5 mm.); overlying skin edematous. There was a small red spot at the most prominent portion of the nodule. Animal still in excellent condition. Feb. 15. Reddening of abdominal nodule had spread peripherally; a tumor of irregular contour and uneven surface was felt under the tense, somewhat edematous skin. The tumor was firm, and the skin was partly adherent to it; there was no softening or fluctuation on palpation. Scarified areas on abdomen showed erythematous dots, but there was no definite change in the appearance of the scarified areas on the eyebrows. The nodules induced by the intradermal injections measured 4×4 mm. The induration was more subcutaneous than intradermal, and at the points of needle insertion the skin was pinkish.

Feb. 19. Abdominal nodule 1.5×2 cm. in size, deep seated, irregular to palpation, and partly adherent to the skin, which appeared somewhat yellowish. Abdomen less edematous. Nodules on eyebrows slightly increased in size; overlying skin normal in color. Feb. 22. Abdominal nodule measured 5×6 cm. and stood out prominently above the abdominal wall. It seemed to consist of several lobulous nodules of varying size. The skin showed a definitely yellowish tint on one side. The lymph glands in the axillary and inguinal regions and at the flexor side of both elbow joints were markedly swollen.

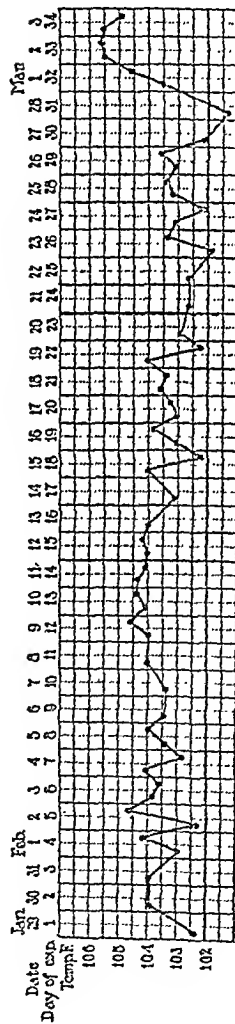
Feb. 23. The abdominal nodule was harder and perhaps somewhat larger (Fig. 13). The lines of scarification on the right abdominal wall were bright red, considerably raised, and indurated (Fig. 9). Erythrocytes 6,234,000, leucocytes 12,000, hemoglobin 90 per cent. The blood yielded cultures of *Bartonella bacilliformis* in a dilution of 1:10,000, and the organism was demonstrated in small numbers in the red cells. Feb. 25. The intradermal nodules on the eyebrows (Fig. 8) were red at the apices. The other lesions had increased in size and intensity.

Mar. 1. The animal had shown no constitutional reaction and appeared to be perfectly well. Erythrocytes 5,120,000, hemoglobin 85 per cent. Cultures of

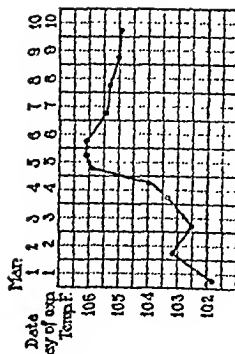
⁶ All the hemoglobin estimates were made with Sahli's hemoglobinometer.



TEXT-FIG. 1. Chimpanzee.



TEXT-FIG. 2. *M. rhesus* 14.



TEXT-FIG. 3. *M. rhesus* 21.

All became attached. The animal had been having irregular fever (104°F. or slightly above) for the past 4 weeks, and the unintentional infection with spotted fever by tick feeding escaped attention for a time. When the animal died on Mar. 4, however, it became evident that death was due to spotted fever, and that *M. rhesus* 21 (Text-fig. 3) and the chimpanzee had received on Mar. 1 Rocky Mountain spotted fever virus as well as *Bartonella bacilliformis*. *M. rhesus* 21 died of typical spotted fever within 10 days (4 days incubation, 6 days of high fever, 106°F. during the first 2 days). Notwithstanding the presence of the spotted fever infection, the blood of Monkeys 14 and 21 both yielded cultures of *Bartonella bacilliformis*, that of *M. rhesus* 21 in a dilution of 1:100,000. Injections into guinea pigs of the blood and of suspensions of the nodular tissue from Monkey 14 induced typical fatal spotted fever.

Spotted Fever in the Chimpanzee.—As the chart shows (Text-fig. 1), the spotted fever infection (Mar. 1 to 15) gave rise in the chimpanzee to a very different temperature curve from that of the pure infection with *Bartonella bacilliformis* (Jan. 29 to Mar. 1). After an incubation period of 5 days the temperature rose to 101°F., then gradually to 102.5°F., then fell to a slightly subnormal level, and remained there for several days. The fever was relatively mild, as compared with that usually observed in spotted fever infection in man, *Macacus rhesus* monkeys, and guinea pigs; in these animals spotted fever gives rise to a temperature of 105–106° lasting 7 to 10 days. The chimpanzee had become inactive on the 5th day, was disinclined to move about, depressed, and indifferent to food. This condition continued for 5 days (until Mar. 9). By this time the accident had become known through the death of *M. rhesus* 14, and 20 cc. of pooled spotted fever antiserum from hyperimmunized rabbits were given intravenously to the chimpanzee and also to the control *rhesus* (No. 21). In the *rhesus* the injection had no effect; the animal died the following day. In the chimpanzee the character of the fever before and after the administration of the serum appeared to be the same, and the recognition of curative effect is difficult unless the short duration of the fever is assumed to have been due to the effect of the serum. In *rhesus* monkeys and guinea pigs, however, the serum has not been known to influence the course of fever unless given during the incubation period.⁷ At all events, the chimpanzee recovered very rapidly and probably without aid of the antiserum.

During the period of spotted fever infection, when the animal was very ill, all the nodules, including the reddened skin lesions, became paler and smaller,

⁷ Noguchi, H., *J. Exp. Med.*, 1923, xxxvii, 383.

undiluted blood positive. Routine examination of blood films failed to reveal blood corpuscles containing *Bartonella bacilliformis*. A portion of the abdominal nodule (Fig. 3) was excised for examination. It showed characteristic proliferation of endothelial cells (Fig. 14), and *Bartonella bacilliformis* was demonstrable in the cytoplasm of the cells (Figs. 5, 15). Cultivation of a suspension of the nodular tissue yielded growth of the organism. A portion of the skin lesion on the scarified area of the abdomen (Fig. 9) was also excised. It was similar in structure to the abdominal nodule (Figs. 10, 11) and yielded a culture of *Bartonella bacilliformis*.

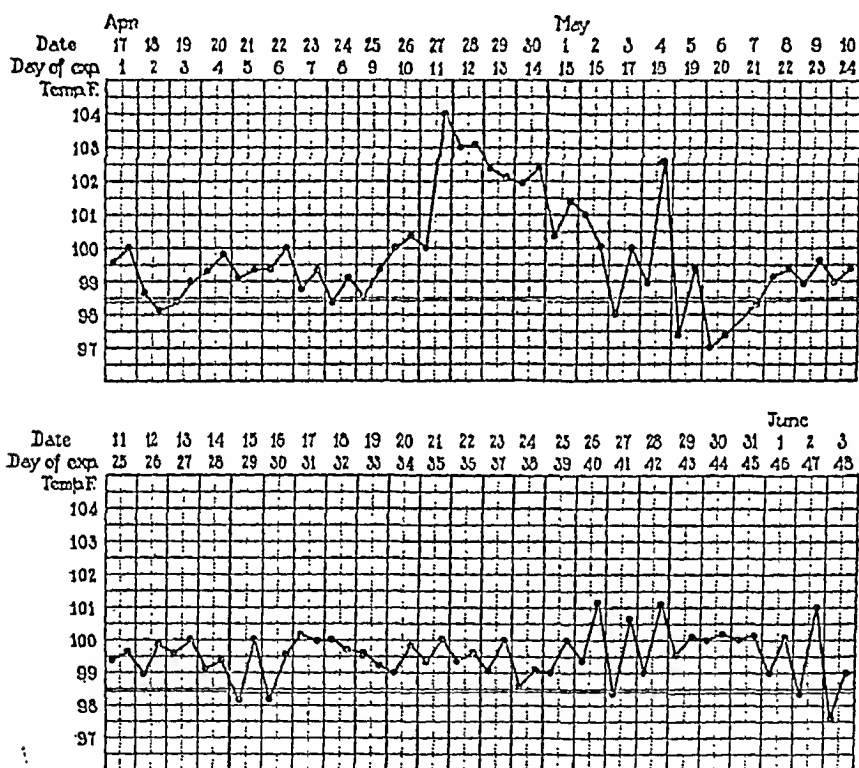
For more than four weeks the blood count, temperature, appetite, and general health and disposition of the animal had remained apparently normal. There were so few microorganisms in the blood on Mar. 1 that undiluted citrated blood in amounts less than 0.2 cc. failed to yield growth, and demonstration of red corpuscles containing *Bartonella bacilliformis* was not possible by the ordinary routine examination of one or two film preparations. The effect of the inoculation had become mainly local, although the lymph glands were still much enlarged.

At this point it seemed desirable to determine the effect of reinoculating the animal with a virulent passage strain from a *rhesus* monkey. *M. rhesus* 14 (Text-fig. 2), which had been inoculated in the same way as the chimpanzee and at the same time, had developed large nodules on eyebrow and abdomen by Feb. 23 (Figs. 16, 20), and the one at the former site had been excised on Feb. 27 for examination. The abdominal nodule (Figs. 21, 22) was excised on Mar. 1, and suspensions of both nodules and of a piece of skin from one of the scarified areas on the abdomen (Figs. 18, 19) were mixed with cultures of *Bartonella bacilliformis* and inoculated intravenously into the chimpanzee. The same material was injected into *M. rhesus* 21 as a test of its virulence.

An accident intervened at this time. *M. rhesus* 14, which had served as the control for the first inoculation of the chimpanzee and was showing far more active and rapidly progressing local and constitutional reactions, had been used in some experiments to determine whether or not *Bartonella bacilliformis* could be transmitted by insects. Thirty ticks (*Dermacentor andersoni*, believed to be free from spotted fever) were allowed to feed on the animal from Feb. 25 to Feb. 27.

The Behavior of Bartonella bacilliformis in the Ourang-Utan.

The response of the ourang-utan to the inoculation of *Bartonella bacilliformis* was similar to that of the chimpanzee, but in this instance there was a moderate febrile reaction (Text-fig. 4) which began 10 days after inoculation and lasted about a week. On the day before the onset of fever *Bartonella bacilliformis* was demonstrable



TEXT-FIG. 4. Ourang-utan.

by culture in a 1:100 dilution of the blood, but a week later the titer of the blood was only 1:10. The local lesions, while characteristic of those induced by the organism, did not approach in severity those usually observed in *rhesus* monkeys. The largest nodules and the slowest to pass away were two which arose subcutaneously at the sites of attempted intravenous inoculation into the basilic vein of the left arm (Fig. 24), and which remained prominent for 2 months.

and the face of the animal was cyanotic, though the color had returned within a week after the time of injection of the serum.

Mar. 1, 1 p.m. Intravenous injection of 1.5 cc. of a mixture of suspension of nodular tissue of *M. rhesus* 14 (removed Feb. 27 and Mar. 1) and cultures of *Bartonella bacilliformis*. *Mar. 5*. The animal ate very little and was inactive. The nodules on the eyebrows were still large (Fig. 12). *Mar. 6*. Temperature 101°F. *Mar. 7*. Temperature 102° a.m., 102.5° p.m. *Mar. 8*. Temperature 101° a.m., 102.5° p.m. Animal inactive, apathetic, appetite poor. The abdominal wounds, where the lesions had been removed, had partly opened, and the surrounding tissues were edematous. *Mar. 9*. Temperature 102.5° a.m., 102° p.m. The animal was very ill. The nodules were pale and cyanotic. Erythrocytes 4,856,000, hemoglobin 80 per cent. 20 cc. spotted fever antiserum given intravenously at 12 noon.

Mar. 10. Temperature 101° a.m., 102.5° p.m. The color had returned to the nodules, and the animal was somewhat more active. Appetite improved. *Mar. 11*. Temperature 102° a.m., 102.4° p.m. The animal was more active, and the color of the face and nodules more nearly normal. *Mar. 12*. Temperature 101° a.m., 101.2° p.m. Eyelids edematous. Erythrocytes 4,288,000, hemoglobin 65 per cent. Blood yielded growth of *Bartonella bacilliformis* in dilution of 1:100. *Mar. 13*. Temperature 101° a.m., 101.8° p.m. The monkey had removed some of the stitches from the wounds caused by excision of the lesions; the wounds were healing satisfactorily, however. *Mar. 14*. Temperature 100.5° a.m., 100.5° p.m. *Mar. 15*. Temperature 99° a.m., 99.5° p.m. Nodules smaller and paler, not cyanotic. Erythrocytes 3,488,000, hemoglobin 60 per cent. Blood culture negative. One of the inguinal glands on the left side was removed for examination. The suspension yielded cultures of *Bartonella bacilliformis* in a dilution of 1:10. *Mar. 17*. Temperature 98.4° a.m., 99° p.m. The animal was very active. Erythrocytes 4,344,000, hemoglobin 65 per cent. *Mar. 20*. Temperature 99.5° a.m., 100° p.m. The remaining nodules had diminished considerably in size and the overlying skin was of normal color. *Mar. 25 to 31*. Temperature normal. The skin lesions had healed and showed slight brownish pigmentation. The site of the abdominal nodule was practically flat, and the induration had disappeared. The nodules on the eyebrows were reddish but small (3 × 4 mm.).

Apr. 2. The nodules on the eyebrows were much smaller. The lymph nodes had also decreased in size considerably. *Apr. 6*. Blood culture negative. Erythrocytes 4,456,000, hemoglobin 75 per cent. *May 1*. The nodules had practically disappeared. Neither the blood nor the lymph yielded cultures of *Bartonella bacilliformis*. *May 3*. Erythrocytes 5,800,000, hemoglobin 85 per cent. The animal had completely recovered.

at sites of inoculation on eyebrows and areas of induration at the sites of the attempted intravenous injections on the left arm.

From this time on the temperature varied within normal limits. On May 11 the erythrocytes numbered 5,632,000, and the hemoglobin was 80 per cent. Blood taken on May 17 yielded cultures of *Bartonella bacilliformis*. The induration at the site of intradermal inoculation on the right eyebrow had disappeared by May 13, but the adjacent lines of scarification had become distinctly raised and reddish, and there was a distinct nodule, 5×6 mm., at the site of intradermal inoculation on the left eyebrow. The area of scarification on the lower right side of the abdomen also showed activity, being markedly raised and reddish. May 20. Distinct nodules on left side of abdomen at sites of inoculation of cultures. One of these was excised, and also a piece of skin from the scarified area on the lower right abdomen. Both showed the characteristic structure of lesions induced on the skin by *Bartonella bacilliformis*, and the organisms were demonstrated in sections of the tissue (Figs. 6, 25, and 26).

May 23. One of the nodules on the left arm measured 2.5×3 cm., the other 5×5 mm. (Fig. 24). The nodule on the left eyebrow was about 8×9 mm., and there were numerous small reddish papules on the scarified area of the right eyebrow (Fig. 23). All the lesions continued to increase in size until about June 1, when the photographs shown in Figs. 23 and 24 were taken. June 2. Blood culture negative for *Bartonella bacilliformis*. Erythrocytes 5,512,000, hemoglobin 80 per cent, leucocytes 11,600. Polymorphonuclears 33.3 per cent, small lymphocytes 45.25 per cent, large lymphocytes 3.8 per cent, large mononuclears 11.0 per cent, transitionals 1.0 per cent, eosinophils 0.7 per cent, mast cells 4.75 per cent.

The monkey scratched the nodule on the left eyebrow until on June 8 she had succeeded in opening it and on June 10 had removed nearly half of it. The remaining portion was excised on June 10 for examination and culture (Fig. 4). The papules on the right eyebrow had become dry and scaly at this time, and the abdominal lesions had practically disappeared.

In the *rhesus* monkey, inoculated with the same material as the orang-utan to serve as a control, the local reactions were less severe than those often observed in this species, but there was a tendency to generalization of the skin lesions, and the systemic reaction was very severe. Within 7 days after inoculation the sites of intradermal injection on the eyebrows were definitely indurated, and there was a slight spontaneous eruption on the abdomen. The temperature rose on the 11th day after inoculation, and there was continuous high temperature ($104-106^{\circ}\text{F.}$) after the 20th day (Text-fig. 5). The hemoglobin at the time of death, 40 days after inoculation, was 15 per cent, and the erythrocytes numbered 1,176,000.

They had disappeared, however, by Aug. 15 (4 months after inoculation).

The ourang-utan (*Pongo pygmaeus*), a young female (probably 2 to 3 years old), was inoculated Apr. 17, 1926. The right eyebrow was scarified and smeared with a piece of nodule just excised from *M. rhesus* 23 and was intradermally inoculated, on the inner aspect, with 0.2 to 0.3 cc. of a suspension of the same tissue. 0.2 cc. of the suspension was injected intradermally into the shaved skin of the right upper abdominal wall, and about 1 cc. was smeared over a scarified area on the right lower side of the abdomen. The left side of the abdomen was inoculated similarly at two sites with a mixture of cultures derived from the blood of *M. rhesus* 7 and *M. rhesus* 18. The mixture included cultures grown on leptospira medium for 10 days and blood agar cultures 6 days old which contained a large number of motile organisms.

The animal received intravenously a mixture containing 6 cc. of the cultures, 1 cc. of the suspension of nodular tissue of *M. rhesus* 23, and 3 cc. of citrated blood from each of two monkeys, *M. rhesus* 11 and *M. rhesus* 24. Two attempts to introduce the syringe needle into the basilic vein of the left arm having failed, the material was inoculated into the corresponding vein of the right arm. A small amount of material from the syringe had, however, been unintentionally introduced subcutaneously into the left arm, and two large subcutaneous nodules later developed at the sites of the attempted intravenous injections.

A blood count made on Apr. 2, 1926, had shown 5,940,000 erythrocytes per c.mm., 12,200 leucocytes, and 85 per cent hemoglobin. The normal temperature of the animal had been taken daily at 11 a.m. and at 4 p.m. for a period of several weeks. It varied from 98.6°F. to 99.6° and was rarely as high as 100° in the afternoon.

Blood taken on Apr. 26, 9 days after inoculation, yielded growth of *Bartonella bacilliformis* in a dilution of 1:100, and a few cells containing the organism were found in film preparations. The erythrocytes at this time numbered 5,392,000, and the hemoglobin was 70 per cent. For the 10 days following inoculation the temperature remained within normal limits, but on the afternoon of Apr. 27 it rose to 104°, and on Apr. 28 it was 103° both morning and afternoon.

Apr. 29. Temperature 102.4° a.m., 102.2° p.m. Appetite poor. Lymph glands enlarged. Slight induration at site of needle puncture on right arm. Erythrocytes 4,880,000, hemoglobin 75 per cent. Apr. 30. Temperature 102° a.m., 102.4° p.m. Animal quiet and apathetic. May 1. Temperature 100.4° a.m., 101.4° p.m. May 2. Temperature 101° a.m., 100° p.m. May 3. Temperature 98° a.m., 100° p.m. Diarrhea. Blood yielded growth of *Bartonella bacilliformis* in dilution of 1:10. Erythrocytes 4,808,000, hemoglobin 80 per cent. Slight induration at sites of intradermal injection on each eyebrow.

May 4. Temperature 99° a.m., 102.6° p.m. May 5. Temperature 97.4° a.m., 99.4° p.m. May 6. Temperature 97° a.m., 97.4° p.m. Distinct nodules

No fluid in the pericardium, no evidence of pericarditis. Heart muscle heavily congested and very hemorrhagic. Liver very pale and soft but showed no gross pathological changes. Spleen normal in size but very dark in color and much harder than normal. Kidneys normal in appearance. There were a few dark bluish enlarged lymph nodes in the mesenterium and one in the large intestine, but otherwise the intestinal tract appeared normal. Axillary and inguinal lymph glands very slightly enlarged. One small nodule on the left eyebrow and another larger one on the back of the left leg. Both on section showed the presence of *Bartonella bacilliformis*.

Erythrocytes 1,176,000, leucocytes 3,200, hemoglobin 15 per cent. Polymorphonuclear leucocytes 50.7 per cent, small lymphocytes 31.1 per cent, large lymphocytes 3.2 per cent, large mononuclears 11.8 per cent, transitionals 1.9 per cent, eosinophils 0.8 per cent, mast cells (basophils) 1.25 per cent, megalo-blasts 1.0 per cent.

The red cells stained very irregularly, a large number of them remaining practically unstained, while about 25 per cent showed marked polychromatophilia. There were a large number of microcytes and a few macrocytes, mostly of irregular shape. No nucleated red cells were found. A few red cells contained *Bartonella bacilliformis*. There were a few threads of fibrin in the smears but no platelets.

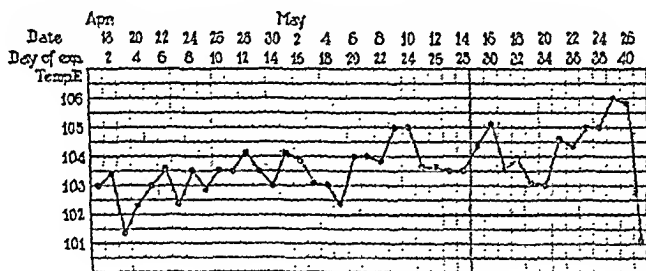
The majority of the polymorphonuclear leucocytes had become so changed that the cytoplasm had practically disappeared. The nucleus was still intact. Most of the lymphocytes were extremely small, about half the size of the red cells.

SUMMARY.

The inoculation of a chimpanzee with cultures and a passage strain of *Bartonella bacilliformis* induced local reactions which, while definite and characteristic, progressed less rapidly and were much less striking than those in the control *rhesus* monkey. *Bartonella bacilliformis* was demonstrated in the blood corpuscles with difficulty, and the fever was slight compared with the high and persistent fever of the *rhesus* monkey. In both the swelling of the lymph glands was an early symptom and constantly present. Definite anemia developed in the chimpanzee only after accidental infection with Rocky Mountain spotted fever and may have been due to either one or both infections, though it disappeared when the blood had become negative by culture for *Bartonella bacilliformis* and the local lesions had disappeared. Incidentally, the chimpanzee was found in this one instance to be less susceptible to the spotted fever than *Macacus rhesus* and guinea pigs.

M. rhesus 37 was injected intravenously (saphenous vein) on Apr. 17, 1926, with the same mixture of cultures and nodular suspension which the ourang-utan had received. The right eyebrow was inoculated intradermally and by scarification with the suspension of the nodule of *M. rhesus* 23, the left in the same way with the mixture used for intravenous injection. The shaved skin of the lower right side of the abdomen was inoculated by scarification with the nodule suspension of *M. rhesus* 23, the lower left side with a suspension of nodular tissue of *M. rhesus* 18. The latter suspension was also inoculated by scarification on two sites on the upper abdomen.

Apr. 23. Slight induration at the sites of intradermal inoculation on the eyebrows. Apr. 24. Indurations on the eyebrows definite; slight spontaneous eruption on abdomen. Apr. 28. Temperature 104.2°F. Distinct nodule at site of intravenous injection on the right leg and red papules at all four sites of scarification on abdomen. Apr. 29. Blood yielded growth of *Bartonella bacilliformis* in dilution of 1:10. Erythrocytes 5,180,000, hemoglobin 70 per cent.



TEXT-FIG. 5. *M. rhesus* 37.

May 1. Temperature 104.2°. May 3. Nodule on left eyebrow 2 × 3 mm. May 7. Lines of scarification on right eyebrow slightly raised. Nodules on left eyebrow and on leg increased in size. May 12. Erythrocytes 5,536,000, hemoglobin 75 per cent. May 19. Blood culture negative.

From May 19 to May 24 this animal was used for the feeding of nineteen ticks (*Dermacentor variabilis*). On May 20 there was a spontaneous papular eruption on the abdomen. The nodules on the eyebrows had not increased in size, but the one on the right leg was 4 × 5 mm. On May 26, when the temperature was 105.8° the animal was used for the feeding of ten bedbugs, which were removed the following morning. May 27. Temperature 101°; animal very ill, cyanotic. May 28. Died.

Autopsy.—Profuse hemorrhage on the abdominal wall and in the omentum but no free blood in the peritoneal cavity. Peritoneal fluid yellowish, slightly turbid, contained a few red cells. Intestines smooth and glistening, no evidence of peritonitis. Lungs whitish gray, extremely anemic, but otherwise normal.

FIG. 10. Section of the lesion shown in Fig. 9, which was excised 32 days after inoculation. Giemsa's stain. $\times 182$.

FIG. 11. The same section at a magnification of 1,000 times, showing the presence of *Bartonella bacilliformis*.

FIG. 12. The appearance of the nodules on the eyebrow of the chimpanzee 31 days after inoculation.

FIG. 13. The abdominal nodule of the chimpanzee as it appeared 25 days after inoculation.

FIG. 14. Section of the nodule shown in Fig. 13, which was excised 32 days after inoculation. Giemsa's stain. $\times 182$.

FIG. 15. The same section at a magnification of 1,000 times, showing the presence of *Bartonella bacilliformis*.

PLATE 27.

FIG. 16. Appearance of the nodules on the eyebrows of *M. rhesus* 14, 23 days after inoculation. Natural size.

FIG. 17. The skin lesions on the abdomen of *M. rhesus* 14, 23 days after inoculation. Natural size.

FIG. 18. Section of one of the skin lesions shown in Fig. 17, excised 23 days after inoculation. Giemsa's stain. $\times 182$.

FIG. 19. The same section at a magnification of 1,000 times, showing the presence of *Bartonella bacilliformis*.

FIG. 20. The abdominal nodule of *M. rhesus* 14, 23 days after inoculation. Natural size.

FIG. 21. Section of the nodule shown in Fig. 20, which was excised 28 days after inoculation. Giemsa's stain. $\times 182$.

FIG. 22. The same section at a magnification of 1,000 times, showing the presence of *Bartonella bacilliformis*.

PLATE 28.

FIG. 23. Appearance of the skin lesions on the eyebrows of the ourang-utan 43 days after inoculation. $2/5$ natural size.

FIG. 24. The large subcutaneous nodules which arose on the left arm of the ourang-utan at the site of unsuccessful attempts to enter the basilic vein at the time of inoculation. Photograph taken 43 days after inoculation. $2/5$ natural size.

FIG. 25. Section of the abdominal nodule of the ourang-utan, excised 40 days after inoculation. Giemsa's stain. $\times 182$.

FIG. 26. The same section at a magnification of 1,000 times, showing the presence of *Bartonella bacilliformis*.

In the ourang-utan, also, *Bartonella bacilliformis* induced a mild systemic and local infection. A rise of temperature occurred 10 days after inoculation, and fever continued for a week, though it was decidedly less severe than that in the control *rhesus*. The lesions induced by scarification were less definite than those which arose at the sites of intradermal inoculation. *Bartonella bacilliformis* was recovered from the blood on the 9th and on the 16th days after inoculation and from nodules excised on the 33rd and 53rd days. A few erythrocytes containing the organism were demonstrated in stained smears, but prolonged search was required to find them.

The symptoms and lesions observed in the chimpanzee and ourang-utan as a result of infection with *Bartonella bacilliformis* are far milder than those of *rhesus* monkeys and show less resemblance to human Oroya fever or verruga.

EXPLANATION OF PLATES.

PLATE 25.

FIG. 1. *Bartonella bacilliformis* in the erythrocytes of *Macacus rhesus*. Selected cells from film preparations of the blood of *M. rhesus* 25 and *M. rhesus* 30. Giemsa's stain. $\times 1,750$.

FIG. 2. *Bartonella bacilliformis* in the erythrocytes of Case S. A. 15, from which the organism was isolated. Giemsa's stain. $\times 1,750$.

FIG. 3. The nodule excised from the abdomen of the chimpanzee 31 days after inoculation. Natural size and color.

FIG. 4. The nodule excised from the eyebrow of the ourang-utan 53 days after inoculation. Natural size and color.

FIG. 5. *Bartonella bacilliformis* in a section of the chimpanzee nodule shown in Fig. 3. Giemsa's stain. $\times 1,750$.

FIG. 6. *Bartonella bacilliformis* in a section of a nodule of the ourang-utan, excised 33 days after inoculation. Giemsa's stain. $\times 1,750$.

FIG. 7. *Bartonella bacilliformis* in a section of a spontaneous nodule of *M. rhesus* 25 excised 23 days after inoculation. For comparison. Giemsa's stain. $\times 1,750$.

PLATE 26.

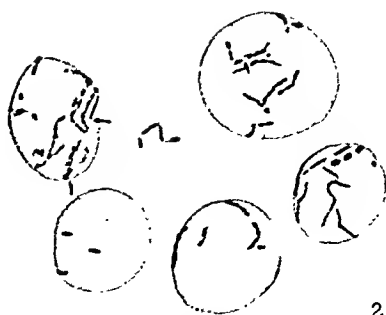
FIG. 8. Appearance of the nodules on the eyebrows of the chimpanzee 27 days after inoculation. $1/2$ natural size.

FIG. 9. The skin lesion on the right side of the abdomen of the chimpanzee as it appeared 25 days after inoculation.



1

Blood of *M. rhesus*.



2

Human blood.



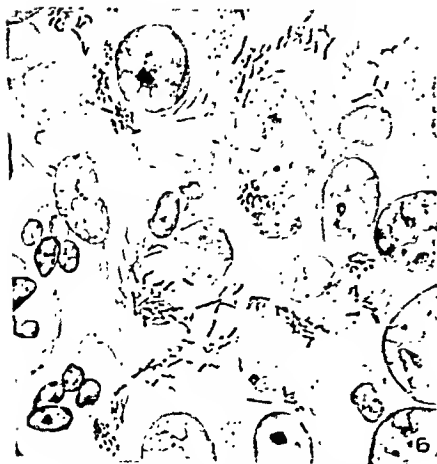
Abdominal nodule, chimpanzee.



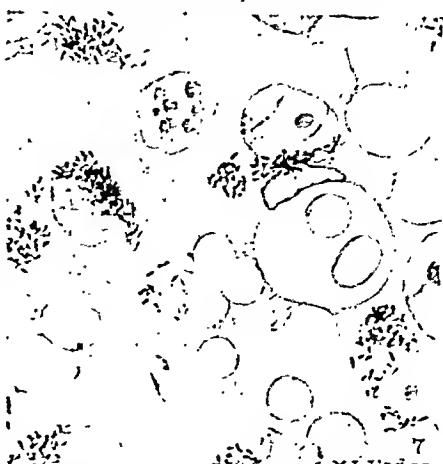
4

Bartonella in chimpanzee nodule.

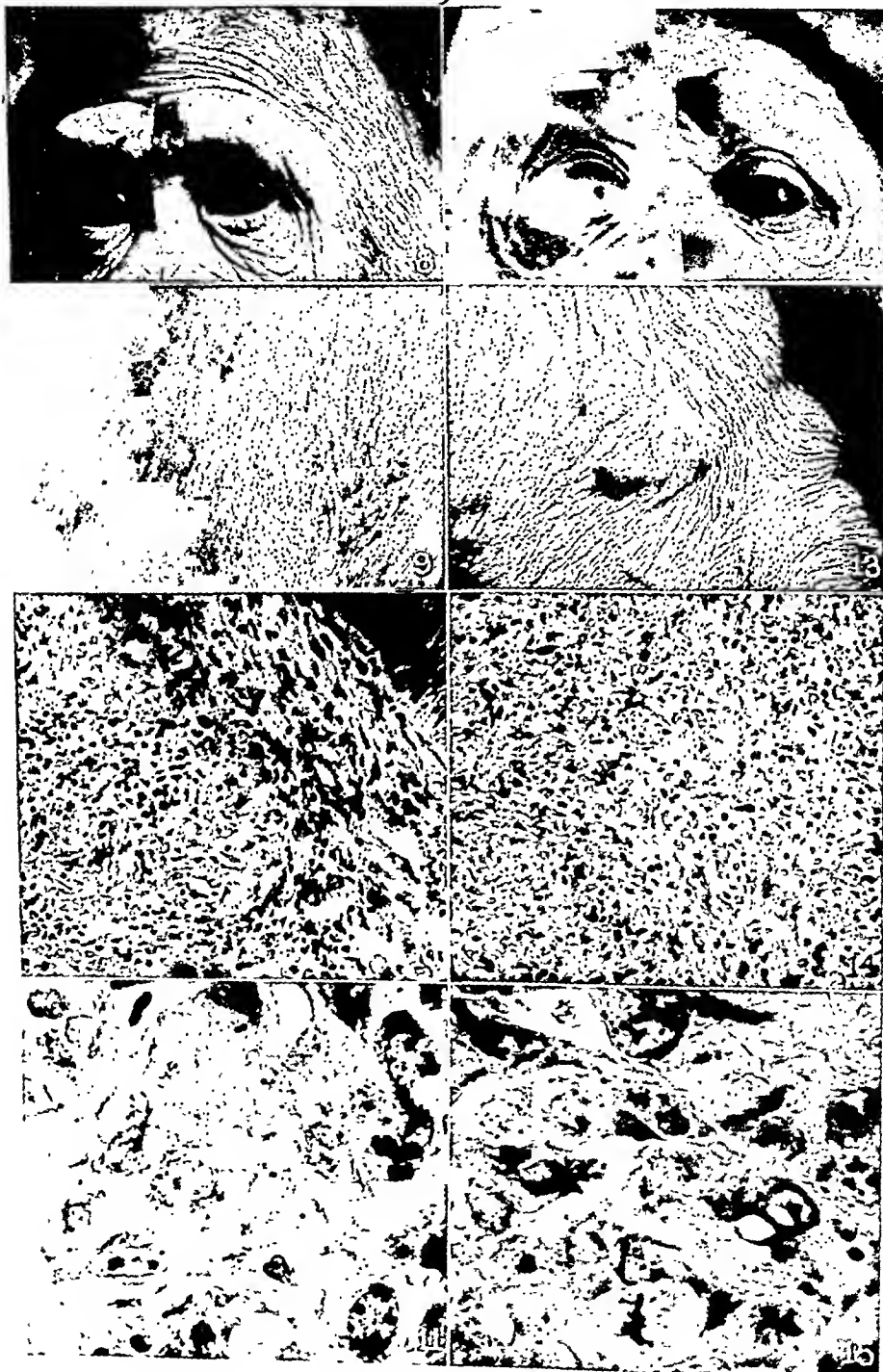
Eyebrow nodule, ourang-utan.

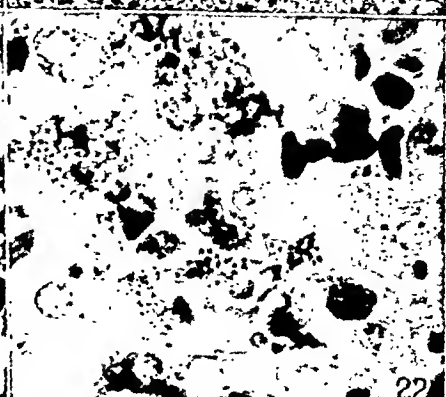
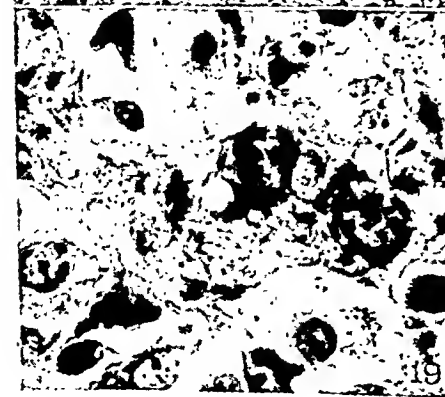
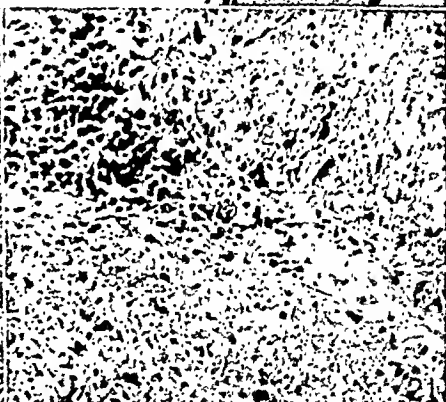
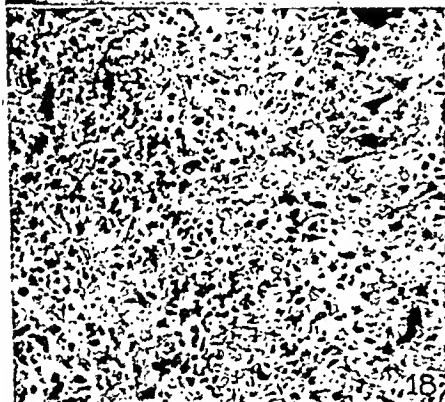


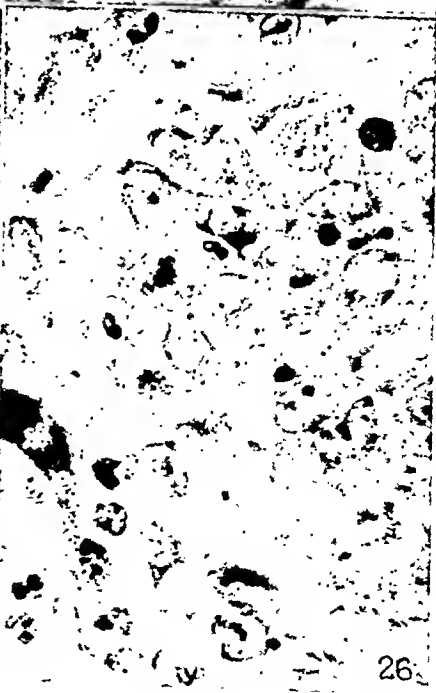
Bartonella in ourang-utan nodule.



Bartonella in nodule of *M. rhesus* 25.







some well known carrier of infectious agents—such, for example, as the tick which transmits Rocky Mountain spotted fever,⁶ tularemia,⁷ and a filterable virus pathogenic for guinea pigs⁸—could also act as a vector of *Bartonella bacilliformis* under experimental conditions. Success in transmitting the parasite under such circumstances, while not solving the problem of the actual vector, would nevertheless furnish a starting point for the investigation of the question.

For the specimens of ticks (*Dermacentor andersoni*) used in these experiments, I am indebted to Dr. R. R. Parker, of the United States Public Health Service, Hamilton, Montana. The ticks were first allowed to feed on normal guinea pigs to determine whether or not they were free from the spotted fever virus. If the guinea pigs escaped infection, the ticks were then placed on the shaved abdominal skin of monkeys (*Macacus rhesus*) in various stages of infection with *Bartonella bacilliformis* and allowed to feed for periods varying from 24 hours to 6 days. Tests of infectiveness were made by feeding the ticks on normal *rhesus* monkeys either immediately following their removal from the infected animal or after an interval of 12 to 14 days.

Two series of experiments were made. In the first, the periods of tick feeding were relatively short—24 hours to 4 days, and several days were allowed to elapse between the removal of the ticks from the infected animals and the test feedings. Infection not being obtained under these conditions, in the second series the period of infective feeding was lengthened to 5 days, the ticks were transferred to normal animals immediately after their removal from the infected ones, and 6 days were allowed for the test feeding. Under these circumstances the ticks proved infective.

Experiment 1 (Negative).

Feb. 1, 1926. Thirty ticks were placed on three infected monkeys (Nos. 7, 12, and 14), ten on each animal, and allowed to remain attached for 1 to 4 days.

⁶ Ricketts, H. T., *J. Infect. Dis.*, 1907, iv, 141.

⁷ Francis, E., *J. Am. Med. Assn.*, 1925, lxxxiv, 1243.

⁸ Noguchi, H., *J. Exp. Med.*, 1926, xlv, 1.

ETIOLOGY OF OROYA FEVER.

V. THE EXPERIMENTAL TRANSMISSION OF *BARTONELLA BACILLIFORMIS* BY TICKS (*DERMACENTOR ANDERSONI*).

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The geographical distribution of Oroya fever, which occurs within certain limited areas of Peru, has suggested to many students of disease that infection is carried by some biting insect or arthropod, and Castellani and Chalmers¹ note particularly the analogous localization of the tick-borne disease, Rocky Mountain spotted fever. Entomological investigation in relation to Oroya fever has not so far implicated any particular insect, with the possible exception of *Phlebotomus verrucarum* Townsend,² and experimental work has been hampered by the lack of animals susceptible to infection with *Bartonella bacilliformis* and methods of cultivating the organism. These obstacles have recently been overcome,^{3,4} however, and the presence of *Bartonella bacilliformis* in insects can now be definitely determined both by cultural methods and by infection experiments. There is considerable evidence,³⁻⁵ moreover, that verruga peruviana is also caused by *Bartonella bacilliformis*, as had long been suspected because of the similar geographical distribution of the two conditions and their frequent association in the same individuals.

It was not possible at the moment to undertake an investigation of insects from the infected districts, but an indirect method of approach to the problem suggested itself, that of determining whether

¹ Castellani, A., and Chalmers, A. J., A manual of tropical medicine, London, 3rd edition, 1919.

² Townsend, C. H. T., *J. Am. Med. Assn.*, 1913, lxi, 1717.

³ Noguchi, H., and Battistini, T. S., *J. Exp. Med.*, 1926, xliii, 851.

⁴ Noguchi, H., *J. Exp. Med.*, 1926, xliv, 697.

⁵ Noguchi, H., and Hercelles, O., *Science*, 1926, lxiv, 121.

abdomen on Feb. 16, 1925, and allowed to remain 4 days. All became attached. The animal showed no signs of infection—no enlargement of the lymph nodes, fever, or skin lesions. Because of prolapse of the rectum, the animal was killed by etherization on Mar. 6, 18 days from the time of tick feeding. Nothing abnormal was found at autopsy, and cultures of heart blood, bone marrow, spleen, lymph nodes, liver, kidneys, testis, and lungs were negative for *Bartonella bacilliformis*.

M. rhesus 20. Inoculated Feb. 16, 1926, intradermally into the shaved skin of eyebrows and abdomen with the mixed suspensions of the viscera of ticks fed on Monkeys 7, 12, and 14. There were small indurations at the sites of intradermal inoculation on the eyebrows after 10 days, but they disappeared within 3 weeks. The abdominal skin showed no reaction to the injections. The temperature rose to 104° or higher (104.6°) on several occasions (Feb. 19, 21, and 24, Mar. 7 and 11). No swelling of the lymph nodes was noted during 42 days of observation.

Experiment 2 (Positive).

Four ticks were allowed to feed on each of two infected monkeys (*M. rhesus* 18 and *M. rhesus* 23) for 5 days, Mar. 24 to 29, 1926.

M. rhesus 18¹ had been inoculated on Feb. 15, 1926, intradermally and by scarification on the shaved eyebrow with a suspension of nodular tissue from *M. rhesus* 5. On Mar. 5 and 18 the blood yielded cultures of *Bartonella bacilliformis* in a dilution of 1:100,000. The nodules on the eyebrows became extremely large. During the time of tick feeding (Mar. 24 to 29) the temperature fluctuated daily between 104° and 105°. On Mar. 27 the blood was positive in a dilution of 1:10.

M. rhesus 23 had been inoculated Mar. 9, 1926, intravenously and by scarification with fourth generation cultures derived from *M. rhesus* 7, and intradermally with a suspension of the nodule excised from *M. rhesus* 18 on that date. The local inoculations induced in time very striking nodules and indurations, and blood taken on Mar. 22 yielded growth of *Bartonella bacilliformis* in a dilution of 1:100,000. The temperature was 104°F. on Mar. 24, when the ticks were placed on the abdominal skin. All were moderately engorged when removed on Mar. 29. The course of infection in this animal subsequently became very severe both locally and systemically. There was a prolonged period of fever, and 2 weeks later spontaneous miliary eruptions appeared on the face. The animal was sacrificed May 1, 42 days after inoculation.

On removal from the infected animals, each lot of ticks was immediately placed on a normal *rhesus* monkey and allowed to feed for a period of 6 days. After the feeding the ticks were dissected, and mixed suspensions of the viscera were inoculated into another normal

M. rhesus 7⁴ had been inoculated intravenously and intradermally on Dec. 21, 1925 (41 days previously), with blood and suspensions of nodular tissue from Monkeys 2, 3, and 4. *Bartonella bacilliformis* was present in the blood 4 days after injection and on several occasions during the following month. The local lesions were well developed by Jan. 19 and were excised* on that date. From Jan. 27 to Feb. 1 fever had been continuous, fluctuating between 104° and 105°F. The ticks were left attached for 24 hours (Feb. 1 to 2). Fever continued until Feb. 9. The blood had yielded cultures of *Bartonella bacilliformis* previously in dilutions as high as 1:10,000,000, but it developed subsequently that the titer had fallen to 1:10 at the time of the tick feeding.

M. rhesus 12 had been inoculated intravenously and intradermally on Jan. 19, 1926, with first generation cultures, derived from the blood of Monkeys 4, 6, and 7, and grown for 14 days on leptospira medium. The blood was positive by culture on Jan. 25 and on Feb. 3 (6 and 15 days after inoculation). The intradermal injections gave rise to small nodules, which, however, never progressed to typical lesions but gradually receded within a month to mere traces of induration. The temperature was continuously high (104°) from Jan. 30 to Feb. 5. The ticks were allowed to feed 72 hours (Feb. 1 to 4).

M. rhesus 14⁴ had been inoculated on Jan. 29, 1926, with a suspension of nodular tissue from *M. rhesus* 3 and with cultures of *Bartonella bacilliformis* derived from human and monkey blood, the material having been introduced intradermally, subcutaneously, and by scarification. The temperature rose after 24 hours, and the animal remained febrile (104-104.6°F.) for 4 days. The ticks were allowed to feed on the animal Feb. 1 and 2. Blood taken on Feb. 8 yielded cultures of *Bartonella bacilliformis* in a dilution of 1:10 only. The local and general symptoms progressed steadily until on Feb. 23 the blood yielded cultures of *Bartonella bacilliformis* in a dilution of 1:10,000, the nodules on the eyebrows were very large and protruding, and those on the abdomen measured 1.5 × 3 cm. It is evident that the ticks were placed on the animal too early in the course of the infection.

Feb. 16, 1926. Three ticks of each lot of ten were placed on the shaved abdominal skin of *M. rhesus* 19, and two of each lot were dissected and suspensions of the viscera inoculated intradermally into the shaved skin of the eyebrows and abdomen of *M. rhesus* 20. The suspensions were tested also by culture. No infection could be demonstrated in the ticks, and the cultures were negative for *Bartonella bacilliformis*.

M. rhesus 19. Nine ticks, of which three had been fed on *M. rhesus* 7, three on *M. rhesus* 12, and three on *M. rhesus* 14, were placed on the shaved skin of the

* All operations were performed under ether anesthesia.

the inguinal and axillary regions on both sides were somewhat swollen. On Apr. 14 one of the inguinal lymph nodes was excised for examination. A suspension of the tissue yielded cultures of *Bartonella bacilliformis*. The temperature was 104–105°F. from Apr. 21 to 25, and blood taken on Apr. 24 yielded cultures of *Bartonella bacilliformis* in a dilution of 1:10. The enlargement of the lymph glands has steadily progressed up to the time of writing (May 30, 1925.) No skin lesions have developed.

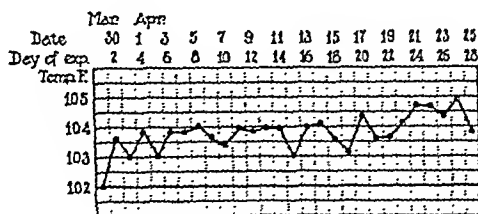
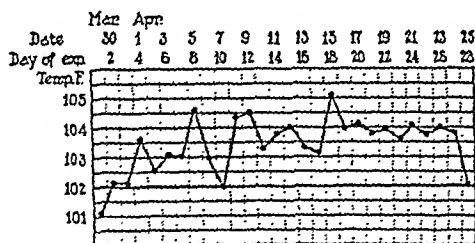
M. rhesus 27. Mar. 29, 1926. Four ticks which had been allowed to feed for 5 days on *M. rhesus* 23 were placed on the shaved skin of the abdomen and allowed to feed for 6 full days. All the ticks became attached and were moderately engorged at the time of removal. The reaction in this animal was much the same as that in the foregoing monkey. Cultures made with a suspension of one of the inguinal lymph nodes excised on Apr. 14 and with blood withdrawn on Apr. 24 were positive in dilutions of 1:10. The animal has shown irregular febrile reactions (Text-fig. 2), but no skin lesions have developed up to the time of writing.

M. rhesus 28 (Text-fig. 3). Apr. 5, 1926, inoculated intradermally on the left eyebrow with a suspension in 1 cc. saline of two ticks fed on *M. rhesus* 26 and on the right eyebrow with a suspension in 2 cc. saline of four ticks fed on *M. rhesus* 27. Six-tenths of a mixture of the two suspensions was injected intravenously. The course of events in this animal was very similar to that in Monkeys 26 and 27. Small nodules appeared at the sites of intradermal injection on the eyebrows about 8 days after inoculation, but they did not progress further. The lymph node removed from the inguinal region on Apr. 14 and blood withdrawn on Apr. 24 both yielded cultures of *Bartonella bacilliformis*. A few papular eruptions had appeared on May 19. The skin became yellowish in color about a month after inoculation and has remained so up to the time of writing.

SUMMARY.

Experiments are reported in which *Bartonella bacilliformis* was transmitted from infected to normal *rhesus* monkeys by the bite of the tick, *Dermacentor andersoni*. A long period of feeding, both on the infected animal and on the normal animal subjected to infection, was required in order to secure positive results. The infection transmitted by the ticks was mild, but definite, as shown by the recovery of *Bartonella bacilliformis* from the lymph nodes and blood.

monkey. Definite, though mild, infection was induced in all the animals, and *Bartonella bacilliformis* was recovered in culture from the blood and lymph nodes. The suspensions of the tick viscera also yielded cultures of the organism.

TEXT-FIG. 1. *M. rhesus* 26.

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W. J. CROZIER

JOHN H. NORTHROP

W. J. V. OSTERHOUT

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CORRECTION.

In Vol. xlv, No. 5, page 614, the third column in Section A of Protocol V should show the death of all of the mice. Two died within 24 hours, two within 48 hours, and the last within 72 hours. The appended diagram giving the correction for this column can be pasted in place on the original protocol.

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The comparative immunological studies of the soluble specific substance and the nucleoprotein of *Pneumococcus* (3) reveal that the soluble specific substance, a nitrogen-free carbohydrate, reacts specifically with antipneumococcus serum of the homologous type. In the dissociated, dissolved, state, it does not serve as antigen; but in the form in which it exists in the cell it functions antigenically to produce type-specific antibodies. The nucleoprotein, on the other hand, is protein in nature and induces in the animal an antiserum which contains only the antiprotein or common, species antibody.

The present study is concerned with the immunological relationships of cell constituents of Friedländer's bacillus and the occurrence of these constituents in culture and body fluids of infected animals.

Methods.

The Soluble Specific Substance.—Methods for the fractionation of soluble specific substance of Friedländer's bacillus have been described in papers from this laboratory (5). It was shown at that time that carbohydrate derived from Strain E (Type B) is dextrorotatory, shows an acid equivalent varying from 670 to 716, is nitrogen-free, and on hydrolysis yields about 75 per cent reducing sugars. It reacts only in type-specific sera to a dilution of 1 to 4 million. Purified, nitrogen-free polysaccharides of Types A and B prepared in this laboratory were utilized in the present study through the courtesy of Drs. Heidelberger and Goebel.

The Nucleoprotein.—Several methods were employed for the separation of the protein and none of the methods were entirely satisfactory. The yield was usually small and the solutions underwent denaturation on keeping. The method finally adopted, however, made use of non-encapsulated cells (since no difference could be shown between the protein derived from encapsulated and capsule-free cells, respectively). The growth from the surface of the agar in Blake bottles was washed off in sterile distilled H₂O. To this suspension NaOH was added to an ultimate concentration of .005 N. The suspensions were frozen and thawed successively a dozen times or more and then diluted 3–4 times with distilled H₂O and centrifuged. The resultant supernatant was filtered, so that a sterile cell-free filtrate was obtained. Precipitation was then effected with a minimum amount of N acetic acid and the precipitate was whirled down. The supernatant was discarded and the precipitate was redissolved in a minimum amount of .01 N NaOH. Usually acid precipitation and solution with alkali were repeated and the final product was made up in .85 per cent NaCl. All protein solutions were standardized on the basis of nitrogen content.

Immunological Reactions.—The method of immunization, the reactions of agglutination and precipitation and protection test have been described in an earlier paper (1).

IMMUNOLOGICAL RELATIONSHIPS OF CELL CONSTITUENTS OF ENCAPSULATUS PNEUMONIÆ (FRIEDLÄNDER'S BACILLUS).

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A previous publication (1) on the biological classification of Friedländer's bacillus reported the existence of at least three specific types and a heterogeneous group among these bacilli. The groupings, designated as Types A, B, and C, and Group X, are sharply defined and highly specific by a number of immunological reactions. In a later communication (2) it was stated that encapsulated strains are usually virulent, produce soluble specific substance, and as antigen, induce the formation of type-specific antibodies which operate effectively both in test-tube reactions and in animal protection tests. Capsule-free strains, on the other hand, are avirulent, do not produce soluble specific substance, and as antigen stimulate only the undifferentiated species antibody. Type-specific antisera react irregularly with capsule-free strains; and the species-specific antisera, while reacting with capsule-free organisms regardless of type derivations, do not react at all with encapsulated cells.

The accumulated evidence on the serological reactions of Pneumococcus (3) and Friedländer's bacillus (1, 2, 4, 5) discloses that both species are composed of specific types which are referable to the elaboration of soluble specific substance by the organisms. Under certain conditions, the cells degrade into capsule-free bacteria which, among other changes, show lack of virulence and capsule formation, loss of elaboration of specific carbohydrate, and loss of type-specific antigenicity—all of which properties are the opposite of those which characterize their encapsulated antecedents. In virtue of the striking parallelism in the immunological behavior of Pneumococcus and Friedländer's bacillus it seemed of interest to project into the latter group the principles which govern the immunological relationships of the cell constituents of Pneumococcus.

TABLE I.

Agglutination of "S" Strains of Friedländer's Bacillus by Anti-P Sera.

Antigen encapsulated strain	Anti-P sera derived from									Anti-S sera—Type			
	Type A			Type B			Group X			A	B	C	X
	1:1	1:5	1:10	1:1	1:5	1:10	1:1	1:5	1:10	1:5	1:5	1:5	1:5
Type A.....	—	—	—	—	—	—	—	—	—	++++	—	—	—
" B.....	—	—	—	—	—	—	—	—	—	—	++++	—	—
" C.....	—	—	—	—	—	—	—	—	—	—	—	++++	—
Group X.....	—	—	—	—	—	—	—	—	—	—	—	—	++++

++++ indicates complete, disc agglutination.

* The figures represent dilution of serum.

TABLE II.

Agglutination of "R" Strains of Friedländer's Bacillus by Anti-P Sera.

Anti-P sera	Protein antigen from	Final dilution of serum							Normal serum 1:5
		1:5	1:10	1:20	1:40	1:80	1:160	1:320	
Type A	Type A	++++	++++	++++	++++	++++	++++	++++	—
	" B	++++	++++	++++	++++	++++	++	++	—
	" C	++++	++++	++++	++++	++	+	+	—
	Group X	++++	++++	++++	++++	++++	++++	++++	—
" B	Type A	++++	++++	++++	++++	++++	++++	++	—
	" B	++++	++++	++++	++++	++++	++++	++	—
	" C	++++	++++	++++	++++	++++	++	++	—
	Group X	++++	++++	++++	++++	++++	++++	++++	—
Group X	Type A	++++	++++	++++	++++	++	+	+	—
	" B	++++	++++	++++	++++	++++	++++	++	—
	" C	++++	++++	++++	++++	++	++	+	—
	Group X	++++	++++	++++	++++	++++	++	+	—

++++ indicates complete agglutination; +++, marked agglutination, ++, moderate agglutination; +, slight agglutination.

bits were immunized to the protein isolated from representative strains of Types A and B and Group X. The immune sera were tested for agglutinins, precipitins, and protective antibodies.

(a) *Agglutinins*.—Antiprotein sera do not contain type-specific

EXPERIMENTAL.

I. The Soluble Specific Substance.—(a) *Antigenic Properties.*—Repeated observations already published from this laboratory leave no doubt that the chemically purified polysaccharide of *Pneumococcus* is non-antigenic. Similar studies (6) of Zinsser, Mueller, and their associates also record the lack of antigenicity of "residue antigen" from a number of bacterial species. The "residue antigen" of these investigators is a substance which is extracted from bacteria and which bears a definite relation to the specific character of the bacterial cell.

In the present study observations on the antigenicity of the carbohydrate of Friedländer's bacillus are confined to the immunization of rabbits with the polysaccharide derived from Group X. In this instance a solution of bacterial cells was prepared from an encapsulated strain and it consequently contained dissociated soluble specific substance. Immunization with this product even in the presence of nucleoprotein yielded no specific antibodies, as will be pointed out later. Since the results from various sources indicate that bacterial polysaccharides are not antigenic, the lack of specific antibody response to a solution containing both protein and carbohydrate is evidence of similar conditions in the case of Friedländer's bacillus, also. At the same time it is clear that bacterial dissolution is accompanied by antigenic dissociation.

(b) *Serological Properties.*—It has been previously demonstrated (1, 2, 4, 5) that the polysaccharides derived from Friedländer's bacillus react specifically with immune sera of the homologous type. (Cf. in this connection Table IV.) In fact, sufficiently conclusive proof has been presented to show that just as has been shown with *Pneumococcus*, the soluble specific substance confers upon the cell its immunological type specificity.

II. The Nucleoprotein.—(A) *Antigenic Properties.*—It is realized that the acetic acid-precipitable material represents more than the nucleoprotein of the bacterial cell, and that it is a mixture of proteins rather than a single antigenic unit. Nevertheless, for the purposes of the present study, this fact offers no difficulty in either the performance or interpretation of the various reactions employed. Rab-

characteristic of the R cells (2) and occurs at a high dilution of serum. Antiprotein sera, in other words, behave in this respect similarly to anti-R sera. It was to be expected, then, that antiprotein sera would agglutinate, also, suspensions of Friedländer's bacilli after decapsulation by Porges' method. Earlier observations (2) pointed out the serological similarity of capsule-free strains obtained by cultural and chemical means. Table III reveals that suspensions of encapsulated cells treated as Porges recommends are agglutinated in antiprotein sera just as are the "R" strains.

TABLE IV.

Precipitation of the Soluble Specific Substance of Friedländer's Bacillus by Anti-P Sera.

Serum	Soluble specific substance of Friedländer's bacillus											
	Type A						Type B					
	2	20	50	100	250	500	2	20	50	100	250	500
Type A (P).....	-	-	-	-	-	-	-	-	-	-	-	-
" B ".....	-	-	-	-	-	-	-	-	-	-	-	-
" C ".....	-	-	-	-	-	-	-	-	-	-	-	-
Group X ".....	-	-	-	-	-	-	-	-	-	-	-	-
Type A (S).....	-	+	++	+++	+	-	-	-	-	-	-	-
" B ".....	-	-	-	-	-	-	+++	+++	+++	+++	+++	+++
Normal.....	-	-	-	-	-	-	-	-	-	-	-	-

+++ indicates compact disc precipitation with clear supernatant; +++, marked disc precipitate; ++, thin film-like scale; +, ground glass turbidity.

The figures represent dilution in thousands.

(b) *Precipitins*.—It has been shown that type-specific precipitins are induced only by the encapsulated cell. Added confirmation of this fact is derived from the observation that antiprotein sera do not react with the specific polysaccharides of Friedländer's bacillus. This is evident from the results presented in Table IV. It is definite that none of the antiprotein sera are able to cause precipitation of carbohydrate isolated from strains of Type A or B.

That antiprotein sera react with capsule-free strains of Friedländer's bacillus constitutes direct evidence of the presence of the species antibody. That the species antibody is in reality an antiprotein

agglutinins for encapsulated strains of Friedländer's bacillus. Evidence for this statement is found in Table I where it is seen that antisera prepared by immunization with protein derived from serologically different strains do not react with the encapsulated cell of either homologous or heterologous type.

It will be recalled that capsule-free (R) strains of Friedländer's bacillus (2) stimulate the formation of the common, undifferentiated

TABLE III.

Agglutination by Anti-P Sera of Suspensions of Friedländer's Bacillus Decapsulated by Porges' Method.

Anti-P serum derived from	Antigen derived from	Dilution of serum				
		1:50	1:100	1:250	2:500	1:1000
Type A	Type A	+++	+++	++++	++++	+++
	" B	++++	++++	++++	++++	+++
	" C	++	+++	+++	++	+
	Group X	+++	+++	++++	++++	+++
	Gran.*	++	++	+	—	—
" B	Type A	++	+++	++++	++++	++++
	" B	++++	++++	++++	+++	++
	" C	++	+++	+++	++	+
	Group X	+++	++++	++++	++++	+++
	Gran.	++	++	+	—	—
Group X	Type A	+	++	++++	++++	++++
	" B	++++	++++	++++	+++	+
	" C	+	+	+++	++	++
	Group X	++	+++	++++	++++	+++
	Gran.	++	++	++	+	—

* This organism is a strain of granuloma bacillus—isolated from an infection of granuloma inguinale.

species antibody; but that they are unable to provoke type-specific antibodies. Accordingly, it seemed pertinent to determine the reaction of antiprotein sera on the non-encapsulated strains. The results of the reactions are given in Table II. It is seen that capsule-free cells derived from any of the serologically different types agglutinate equally well in all the antiprotein sera. The agglutination is

antibody is evidenced by the reaction of protein precipitation in antiprotein sera. The results of these reactions are presented in Table V. It is seen that sera prepared against protein isolated from three serologically different strains cause the precipitation of protein derived from any of the four different strains. It becomes obvious therefore that nucleoprotein induces the formation of species antibodies which cause agglutination of capsule-free cells and precipitation of protein and that the reactions exhibit none of the type relationships.

In addition, Table V reveals that antiprotein sera of Friedländer's bacillus reacts with protein derived from *B. aerogenes*, *B. coli*, and granuloma bacillus. Stated in another way the protein of Fried-

TABLE VI.

Protection Offered by Anti-P Sera against Infection with Friedländer's Bacillus (Type A).

Type A encapsulated culture	Antiserum derived from				Virulence controls
	Type A		Type B		
	Amount	Result	Amount	Result	
cc.	cc.		cc.		
.001	.2	D. 20	.2	D. 19	
.0001	.2	" 16	.2	" 15	
.00001	.2	" 39	.2	" 16	D. 24
.000001	.2	" 39	.2	" 43	" 39
.0000001					" 65

D. indicates death, the numerals representing the number of hours before death occurred.

länder's bacillus bears a definite serological relationship to proteins of closely allied species. This lends considerable assistance in the interpretation of the results of former investigators who have observed that anti-Friedländer sera caused agglutination of *B. rhinoscleromatis* (7-10), *B. aerogenes* (11), typhoid (12), and granuloma bacillus (13), etc. The explanation of such cross-agglutinations appears to depend upon the fact that immunization with non-encapsulated strains or prolonged immunization with encapsulated strains stimulates the formation of agglutinins which act not only on R cells

TABLE V.

Precipitation by Anti-P Sera of the "Nucleoprotein" of Friedländer's Bacillus and Allied Organisms.

Antigen*	Anti-P serum from	Ultimate dilution of protein						
		200	1000	2000	4000	8000	16,000	32,000
Type A protein	Type A	+++	+++	++	++	+	±	—
	" B	+++	+++	++++	+++	+++	+++	++
	Group X	+++	+++	++	+	—	—	—
Type B protein		500	2500	5000	10,000	20,000	40,000	80,000
	Type A	++++	++++	+++	++	+	±	—
	" B	++	++	+++	+++	+++	+++	+
Type C protein	Group X	+++	+++	++	+	±	—	—
		200	1000	2000	4000	8000	16,000	32,000
	Type A	++	++	++	+	+	±	—
Group X protein	" B	++	++++	++++	++++	+++	+++	+
	Group X	—	+	++	++	+	—	—
		500	2500	5000	10,000	20,000	40,000	80,000
<i>B. aerogenes</i> protein	Type A	++++	++++	+++	++	+	±	—
	" B	++	++	+++	+++	++	++	±
	Group X	++	++	+	+	±	—	—
<i>B. coli</i> protein		500	2500	5000	10,000	20,000	40,000	80,000
	Type A	++	+	+	±	—	—	—
	" B	++	+	+	±	—	—	—
Granuloma bacillus protein	Group X	++	+	±	—	—	—	—
		300	1500	3000	6000	12,000	24,000	48,000
	Type A	++	+	+	±	—	—	—
	" B	++	++	+	+	±	—	—
	Group X	++	++	+	+	—	—	—
		200	1000	2000	4000	8000	16,000	32,000
	Type A	++	++	+	+	—	—	—
	" B	++	++	++	+	+	—	—
	Group X	+	+	±	—	—	—	—

++++ indicates heavy precipitation, supernatant clear; +++, marked precipitation with cloud; ++, marked cloud, no precipitation; +, cloud; ±, faint cloud.

* None of the proteins were precipitated by normal serum.

actually the case is borne out by the data given in Tables VI and VII. The lack of any protective action by antiprotein sera against infection with virulent strains of homologous and heterologous types is striking.

(b) *Serological Properties.*—

1. *Precipitation of Protein in Anti-S Sera.*—Anti-Friedländer sera resulting from immunization with encapsulated strains are dominantly type-specific. Consequently anti-S sera contain negligible amounts

TABLE IX.

Precipitation by Anti-R Sera of the "Nucleoprotein" of Friedländer's Bacillus.

Antigen	Anti-R serum from	Dilution of protein						
		200	1000	2000	4000	8000	16,000	32,000
Type A protein	Type A	+++	+++	++	++	+	—	—
	" B	+++	++	++	+	—	—	—
	Group X	++	+	+	±	—	—	—
Type B protein		500	2500	5000	10,000	20,000	40,000	80,000
	Type A	+++	++	+	—	—	—	—
	" B	++	++	+	—	—	—	—
	Group X	++	+	+	—	—	—	—
Type C protein		200	1000	2000	4000	8000	16,000	32,000
	Type A	++	+	+	—	—	—	—
	" B	++	+	+	—	—	—	—
	Group X	++	+	+	±	—	—	—
Group X protein		500	2500	5000	10,000	20,000	40,000	80,000
	Type A	+++	++	+	—	—	—	—
	" B	++	++	+	—	—	—	—
	Group X	+++	+++	++	+	—	—	—

of antiprotein as has been pointed out previously (2) by the agglutination of R cells in anti-S sera. In virtue of the presence of antiprotein, such sera may cause precipitation of protein. This fact is illustrated in Table VIII. Type A anti-Friedländer serum definitely contains the species antibody, while the remaining type sera contain traces or none at all.

2. *Precipitation of Protein in Anti-R Sera.*—Capsule-free strains of Friedländer's bacillus are agglutinated in antiprotein sera (Table

of Friedländer's bacillus, but on R cells of closely related species, as pointed out above.

III. Protection.—Immune sera prepared by immunization with encapsulated Friedländer's bacilli confer upon white mice specific protection against infection by strains of the homologous type (1).

TABLE VII.

Protection Offered by Anti-P Sera against Infection by Friedländer's Bacillus (Type B).

Type B encapsulated culture	Anti-P serum derived from				Virulence controls
	Type A		Type B		
	Amount	Result	Amount	Result	
cc.	cc.		cc.		
.001	.2	D. 16	.2	D. 15	
.0001	.2	" 16	.2	" 15	
.00001	.2	" 39	.2	" 20	D. 16
.000001	.2	" 39	.2	" 67	" 39
.0000001					" 39

D. indicates death, the numerals representing the number of hours before death occurred.

TABLE VIII.

Precipitation of the Nucleoprotein of Friedländer's Bacillus by Anti-S Sera.

Anti-S Friedländer sera	Friedländer protein derived from							
	Type A		Type B		Type C		Group X	
	*600	6000	1200	12,000	900	9000	500	5000
Type A.....	++	+	+	++	++	+	++	+
" B.....	+	—	+	—	±	—	+	—
" C.....	++	+	++	+	++	+	++	+
Group X.....	+	—	++	—	+	±	+	—

* These figures represent the dilution of protein.

Immunization with non-encapsulated cells, however, yields no protective antibodies (2). Protective substances, therefore, accompany type-specific antibodies. It was anticipated then that lacking type-specific agglutinins and precipitins, Friedländer antiprotein sera would afford no passive protection against active infection. That this is

strain. It was found that removal of agglutinins by one strain adsorbed the agglutinins for the remaining R strains also. Moreover, the loss of agglutinins by adsorption was accompanied by a loss of precipitins. A typical protocol is presented in Table X. It is clear that adsorption of a serum resulting from immunization with protein derived from a strain of Type A, for example, with any of the four "R" strains deprives the serum of precipitins for protein derived from any of the types. It appears therefore that the undifferentiated species antibody is the same antibody whether it occurs in anti-R or antiprotein sera.

TABLE XI.

*Occurrence of Soluble Specific Substance in Culture Filtrate.
Friedländer Bacilli.*

Strain	Dilution of filtrates											
	After 4 hrs.				After 8 hrs.				After 12 hrs.			
	1:1	1:5	1:20	1:50	1:1	1:5	1:20	1:50	1:1	1:5	1:20	1:50
Type A.....	+	-	-	-	+	+	-	-	+++	++	+	-
" B.....	-	-	-	-	±	-	-	-	+	+	-	-
" C.....	+	-	-	-	++	+	-	-	++	++	+	-
Group X.....	±	-	-	-	++	+	-	-	++	+	-	-

*Occurrence of Soluble Specific Substance in Culture Filtrates of
Friedländer's Bacillus.*

It has been shown by Dochez and Avery (14) that the soluble specific substance of *Pneumococcus* is demonstrable in culture filtrates, and that the progressive increase of the carbohydrate bears a striking relation to the growth curve of the culture. Their results show clearly that the soluble specific substance is a product of metabolic activity rather than a product of cell disintegration.

In similar fashion, the polysaccharide of Friedländer's bacillus is demonstrable in actively growing cultures. Cell-free filtrates obtained at different intervals during the growth are precipitated specifically by anti-Friedländer sera. The data submitted in Table XI show that in some instances specific carbohydrate is present in filtrates as early as 4 hours after growth has been initiated. The

II). Evidence of the reciprocal nature of this reaction was obtained in the precipitation of protein by anti-R sera. It is seen from the results presented in Table IX that anti-R sera cause the precipitation of protein from Friedländer's bacillus of the different types. The conclusion can be drawn, therefore, that the common species antibody reacts with the undifferentiated antigen of Friedländer's bacillus whether the antigen is in the form of non-encapsulated cells or in the form of dissolved protein.

TABLE X.

Precipitation of Friedländer Protein by Anti-P Sera after Adsorption of the Species Antibody by R Strains.

Antigen protein derived from	Anti-P serum (Type A) after adsorption by R strains derived from encapsulated strains of								
	Type A			Type B			Type C		
	1:200	1:2000	1:8000	1:500	1:5000	1:10,000	1:200	1:2000	1:8000
Type A.....	—	—	—	—	—	—	—	—	—
" B.....	—	—	—	—	—	—	—	—	—
" C.....	—	—	—	—	—	—	—	—	—
Group X.....	—	—	—	—	—	—	—	—	—

* The figures represent the dilution of protein.

(c) *Precipitation of Protein in Antiprotein Sera.*—It has been shown above that protein derived from any of the serological types is precipitated in all antiprotein sera. (Cf. Table IV.)

Adsorption of Antibodies in Antiprotein Sera by Non-Encapsulated (R) Strains of Friedländer's Bacillus.

Because agglutination of R cells occurs in antiprotein sera, and precipitation of protein is obtained in anti-R sera, experiments were conducted to gain information concerning the identity of the antibody involved in both reactions. Each antiprotein serum was adsorbed with heat-killed suspensions of R cells derived from encapsulated strains of Types A, B, and C, and Group X. Adsorption was continued until all the agglutinins were removed for the adsorbing

Occurrence of Soluble Specific Substance in Friedländer Infections.

The soluble specific substance of *Pneumococcus* has been demonstrated in the serum and urine of patients during pneumonia by Dochez and Avery (14). Blake (15) has shown that this is also a fact in pneumonia due to Friedländer's bacillus. This is the only reference of its kind concerning Friedländer infection which has come to our attention. In the present study experiments were performed to detect specific carbohydrates in rabbits infected by intraperitoneal injections of Friedländer's bacilli. The urine and blood of the infected rabbits were collected and tested for soluble specific substance

TABLE XIII.

Occurrence of Soluble Specific Substance in the Blood and Urine of Animals Infected with Friedländer's Bacilli.

Type of infection	Body fluid	Dilution of fluid		
		1:1	1:5	1:10
Type B	Serum	++	+	—
	Urine	+	—	—
" C	Serum	+++	++	+
	Urine	++	+	—

The precipitin reaction was obtained only in homologous immune serum.

by the usual precipitin technique. It is seen from Table XIII that the specific polysaccharide of Friedländer bacilli is present in both urine and serum of rabbits, and is demonstrable within 18 hours after infection.

DISCUSSION.

The soluble specific substance of Friedländer's bacillus endows the cell with type specificity, and is separable from the bacterial cell as a pure, nitrogen-free polysaccharide. When dissociated from the cell, it does not function as antigen, but in the form in which it exists in the cell it stimulates the formation of antibodies which cause type-specific agglutination of encapsulated cells, precipitate the carbohydrate derived from organisms of the homologous type, and afford

amount of the carbohydrate increases rapidly so that after 24 hours' growth a definite reaction may be obtained in culture filtrate in dilution of 1:20, and in one case (Type A) even in a dilution of 1:50.

Occurrence of Protein in Culture Filtrates of Friedländer's Bacillus.

The type specificity of anti-Friedländer sera depends in part upon the integrity, and the absence of R cells in the culture used for immunization. It becomes of importance, therefore, to determine the rapidity of the disintegration of Friedländer's bacilli. Dissociation may be estimated by the presence of the common or protein antigen. Accordingly an analysis of the protein content of culture filtrates of Friedländer's bacillus was made by the usual protein pre-

TABLE XII.

Occurrence of Protein in Culture Filtrates of Friedländer Bacilli.

Strain	Dilution of filtrates											
	At 12 hrs.		At 24 hrs.		At 72 hrs.		5 days		9 days			
	1:1	1:10	1:1	1:10	1:1	1:10	1:1	1:10	1:1	1:5	1:10	1:20
Type A.....	—	—	—	—	—	—	—	—	+	±	—	—
" B.....	—	—	—	—	—	—	—	—	+++	+±	+	—
" C.....	—	—	—	—	—	—	—	—	+++	+	±	—
Group X.....	—	—	—	—	—	—	—	—	+++	+	±	—

cipitation test. As brought out in Table XII, no protein was demonstrated in culture filtrates after 5 days' growth. On the 9th day, precipitation of protein was obtained in all the filtrates studied. In contrast to the carbohydrate which is elaborated during the period of active growth, the protein is demonstrable after this period and when cell disintegration takes place.

This fact becomes of great significance in immunization with suspensions of Friedländer's bacillus. Despite the fact that both disintegration and R cells are absent in the cultures used for immunization, anti-Friedländer sera may contain variable amounts of protein antibody. This is evidence that the body defenses not only engender type-specific antibodies, but also include a mechanism which causes a cleavage or disintegration of the specific antigen.

CONCLUSIONS.

1. The soluble specific substance of Friedländer's bacillus is non-antigenic when dissociated from the cell. It is different for each type and it is highly reactive in the corresponding anti-S serum.

2. The nucleoprotein is antigenic, induces the species or protein antibody which reacts with capsule-free cells and protein derived from all types. Antiprotein sera do not react with either the encapsulated cell or the polysaccharide derived from it, and they offer no protection against infection.

3. Anti-R and antiprotein sera are identical in their behavior.

4. The carbohydrate of Friedländer's bacillus is demonstrable in filtrates of actively growing cultures and in the blood and urine of infected animals.

5. The protein is demonstrable in filtrates of only old, disintegrating cultures.

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passive protection in white mice against infection by bacilli of the same type. As reacting substance, it is precipitated only in antisera resulting from immunization with encapsulated strains from which it is derived.

The nucleoprotein is separable from dissolved Friedländer's bacilli by precipitation with acetic acid in the cold. This constituent differs in nature and in serological behavior from the soluble specific substance. It is protein in nature and is a common, undifferentiated constituent of all types. It is antigenic and provokes in the animal the common protein or species antibody. The species antibody does not react with encapsulated bacilli of any type nor with the soluble specific substance of either homologous or heterologous types, and does not protect against infection with Friedländer's bacillus. Anti-protein sera, however, cause agglutination of capsule-free cells derived from any of the serological types by either cultural or chemical methods; and they react also with protein from all types. Moreover, the protein antibody is of a sufficiently general nature to react with protein from allied bacteria. In this fact resides the explanation for the confusing cross-agglutination reactions obtained with related organisms by former workers. That the protein antibody is of more or less common occurrence among other species of bacteria gains evidence from the results of numerous investigators. The studies from this laboratory show this with *Pneumococcus*, and the work of Lancefield (16), Hitchcock (17), and Tunncliffe (18) discloses a distinct serological relationship between various species of the Gram-positive cocci. The contributions of Dopter (19) and Eberson (20) depict similar relationships among the Gram-negative cocci, and Smith and TenBroeck (21) and Felix (22) offer comparable data for members of the typhoid-colon group.

Antisera prepared by immunization with protein or with a degraded non-encapsulated R culture contain antibodies which are identical in their immunological reactions.

The presence of soluble specific substance in filtrates of growing cultures suggests that it is a product of growth activity of the cell. The presence of protein in filtrates of old cultures only, indicates that it is a product of cell autolysis and disintegration.

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and of far greater potency for free antibody production than *Bacillus tuberculosis*.

The premises from which the work reported in the present paper takes its departure, some of them elucidated by our associates and ourselves, many of them either suggested or independently worked out by others, may be briefly stated as follows:

1. The tuberculin reaction is independent of general anaphylaxis to tuberculo-protein. (This was quite clear from our early paper (1), which confirmed by other methods the suggestions made long before by Baldwin (2) and by Krause (3), and confirmed also by Selter (4) and by Bessau (5).)

2. Tuberculin hypersensitiveness in its typical and extreme form cannot be induced by dissolved extracts of the tubercle bacillus provided that these are filtered through Berkefeld filters to remove formed particles, but it can be characteristically induced not only by infection with the living bacilli, but by treatment with dead organisms, even when these are boiled (6). These facts confirm the importance of typical tissue reactions spoken of as tubercles in the mechanism of tuberculin sensitization.

3. The mechanism of tuberculin reactions is independent of the presence in the animal body of those precipitating or agglutinating antibodies which act either upon the whole bacilli or on the precipitable carbohydrate "residue" antigen *in vitro*.

4. Conversely, the substance in tuberculin which is responsible for reaction in the sensitized animal is chemically separable from the precipitable residue carbohydrate fraction which reacts with antibodies *in vitro*, and is probably a nitrogenous substance, perhaps a form of protein (Mueller (7); Laidlaw and Dudley (8)).

There are many other minor facts which we have emphasized in preceding papers and which might be included in the premises from which we have worked, but these have been discussed by us in other places and will appear, where important, in other parts of this communication.

The difficulties of working with bacterial allergy have been manifold, chiefly because the bacterial cell is chemically complex, often possessing primary toxicity which produces reactions that are not easily distinguished from allergy in the sensitized.

AN EXPERIMENTAL ANALYSIS OF BACTERIAL ALLERGY.

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PLATES 29 AND 30.

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I.

In the study of hypersusceptibilities a differentiation has been gradually developed between the phenomena concerned in true anaphylaxis to the coagulable proteins, in which it is generally assumed that a true antigen-antibody union upon the cells of the body underlies the responsible mechanism, and the many other manifestations of hypersusceptibility in which no such specific antigen-antibody relationship has been demonstrated. In the case of the bacterial hypersusceptibilities it is quite generally accepted that true protein anaphylaxis plays a relatively unimportant rôle in the occurrences of spontaneous infection, but there are other manifestations, spoken of as bacterial allergy, which are recognized both by systemic and local reactions, and which are of considerable importance diagnostically and perhaps pathologically and prognostically. The mechanism of these conditions has not so far been clearly understood.

In our own studies we have occupied ourselves for a number of years chiefly with the tuberculin reaction, since this phenomenon represents the most extreme example of bacterial allergy and is easily amenable to experimental study. Since, however, we encountered considerable experimental difficulties in elucidating the mechanism of the tuberculin reaction by direct attack, we were forced to extend our studies to experiments with many different bacteria, choosing, in addition to tubercle bacilli and their products, such organisms as the staphylococcus, streptococcus, typhoid bacillus, *Bacillus abortus bovis* and pneumococcus, thus including organisms of many degrees of solubility

injected. We believe that many erroneous interpretations have resulted from this. Moreover, we believe that in itself this non-specific irritability of the skin, whatever its mechanism, following large parenteral injections of protein material may have considerable significance in skin reactions in general. Incidentally, the recent papers of E. F. Müller (9), on the profound influence of any intracutaneous injection upon the involuntary nervous system and the capillary mechanism, furnish a clue to the explanation of these occurrences.

For all these reasons we feel that it is of great importance to define the different types of skin responses to the injection of bacterial substances, although these have often been looked upon merely as quantitative differences in the intensity of one and the same mechanism. The following types of reaction may occur:

1. The purely toxic reaction which has no direct connection with allergy, and which is due to the primary toxicity of the bacterial materials. Examples of this are the Schick and Dick tests which may be indistinguishable from true allergic reactions.

2. The immediate urticarial skin reaction which appears within a short time is chiefly characterized by edema with little inflammation and fades promptly. This is the reaction which, in man, may be regarded as indicating protein sensitization, and which plays a minor rôle in some of the phenomena of bacterial allergy in guinea pigs.

3. The delayed skin reaction which usually does not become manifest in less than 5 or 6 hours, reaches its highest development within 18 to 48 hours, is characterized not only by edema, but by inflammatory changes, and which, by slowness of complete fading, indicates that more has occurred than a simple edema. These reactions are usually well demarked, like an erysipelas, but do not go on to central necrosis or the central hemorrhages. This is the common type of reaction obtained both in man and guinea pigs with bacterial products and, as we shall see, sometimes occurs with protein sensitization.

The classical tuberculin and abortin reactions probably consist, in part, of reactions of the preceding class but, superadded upon these, there is tissue injury with hemorrhage and sometimes necrosis. Although these reactions may be regarded as quantitatively intensified examples of the preceding, the frequency of severe central cell injury and necrotic changes in relatively small reactions of this type, as contrasted with the absence of these even in large reactions of the pre-

Also, there have been difficulties in the choice of the proper animals for experimental purposes in that, here even more than in protein anaphylaxis, fundamental differences exist in the mechanism by which different species of animals respond to sensitization. The primary toxicity of many of the bacterial materials, and the necessity of often working with suspensions rather than with true solutions, render unreliable intravenous injection, with constitutional allergic responses, and we have preferred to confine our studies chiefly to skin reactions. This is, of course, not absolutely dependable, since responses of the skin need not necessarily run parallel to general hypersusceptibility. But accumulating clinical evidence seems to indicate that such a parallelism is fairly close, and if we remember possible divergences under special circumstances, this method need not lead us into error.

In choosing the proper animals for the experiments, the primary purpose of elucidating bacterial allergies as they occur in man naturally inclines one to choose that animal in which skin allergy is apt to be similar to that occurring in man. We have worked with guinea pigs as fulfilling this purpose more closely than most other animals, combining with this great convenience of experiment. But it is important to remember that with allergy as with true anaphylaxis, things which are true of guinea pigs are often quite inapplicable to other animals, notably rabbits. Reactions such as delayed bacterial allergy and the typical tuberculin reaction follow in guinea pigs very much the same rules, as to time and appearance, which are observed in man, though probably with considerably less quantitative delicacy. In favour of rabbits, of course, is the fact that the Arthus phenomenon which cannot easily or typically be elicited in guinea pigs occurs in rabbits, and we believe that in man the hastened reaction occurring upon subcutaneous injection of diphtheria antitoxin and the often disturbing reactions following the later injections of rabies vaccine are closely analogous to the Arthus phenomenon. On the whole, however, we believe that observations made upon guinea pigs by intracutaneous reactions will be most directly useful; and after this, it may perhaps be of some value to "cross-index" these facts with other animals.

In the observation of skin reactions of guinea pigs, there are many pitfalls which have led to a good deal of confusion to us and, we believe, to others, and before describing the experimental results it will pay to consider these, since no adequate observation can be made without taking them into consideration.

In the first place, it is necessary to evaluate the primary toxicity of the bacterial materials worked with, since thereby allergic skin effect becomes merely comparative by reason of the inflammatory reactions produced by the substance on the normal animal. In the second place, there are variations in individual guinea pigs in their reaction to bacterial materials, sometimes because of pregnancy, at other times perhaps because of infectious processes previously sustained. The most serious source of error is a non-specific hypersusceptibility which is not always, but usually developed in guinea pigs 8 to 14 days after the injection of massive amounts of any foreign protein. Such injections seem to induce a non-specific change of reaction capacity which gives rise to moderate but confusing inflammatory reactions when bacterial or other substances are subsequently

On May 20 the animals were then again tested with this O.T. 1-10 and the same abortin 1-5 with the following results:

	O.T.	Abortin
1.	—	Severe +++++ with central white spot
2.	+	+++
3.	++	Strong +++
4.	+	+++

In these experiments it would appear that the specificity is a fairly strict one. As allergy becomes more extreme, however, the overlapping becomes more marked, as the following protocol of another set of animals done at a time when necrotic centres and hemorrhagic reactions were more violent illustrates.

	Abortin	O.T.
1. <i>B. abortus</i> -sensitized.....	+++	±
2. " " "	+	—
3. " " "	+++++	+++
4. " " "	++	+++
5. Tuberculin-sensitized.....	±	+++
6. " " "	—	++

In general it may be stated that *abortus* animals must remain in fairly good condition and resist infection without too rapid an emaciation. Otherwise they remain negative in the same way as a tuberculous animal in the prelethal stages. Given favourable conditions, however, they become positive about 8 days after infection.

The following experiment is added because it illustrates the same relative specificity with *Bacillus abortus* and staphylococcus animals, and further indicates the interesting fact which we consider of some importance, that with sufficient treatment animals may be rendered abortin-sensitive by injections of dead *Bacillus abortus* suspensions. This is again in contradiction of former opinions that only living bacilli can render such animals allergic, and in this way parallels the observations referred to above on analogous conditions with tuberculin sensitization. In this case the animals were prepared by 6 to 9 injections each of suspensions of *abortus* bacilli killed at 65° in the water bath. The tests here reported were done 3 weeks after the last injection.

ceding class, suggests that there is in addition a factor involved in the hemorrhagic-necrotic ones which is absent in the former.

This classification is a tentative one, of course, since much about the mechanism of skin reactions is not yet clear, and we submit it as a working basis.

II.

Specificity of Bacterial Allergy.

It is, of course, of the greatest importance to determine from the beginning the degree to which the bacterial allergic phenomena with which we are dealing are specific. Specificity, apart from being a biological phenomenon of the greatest practical importance, furnishes a definite clue to mechanism, and much of the clinical experience of those engaged in doing skin reactions upon patients, as well as investigations in animals, has left much uncertainty regarding this question.

The following experiments, carried out with tubercle bacilli and various strains of *Bacillus abortus bovis* kindly furnished us by Dr. Theobald Smith, serve to define these relations.

The animals listed below were sensitized with living *Bacillus abortus* and subsequently tested by intracutaneous inoculations with O.T. and abortin produced from Bacillus 1211 by the method used to produce O.T.

1. Living	<i>Bacillus abortus bovis</i>	Feb. 17.
2. " 1211	" "	Apr. 6.
3. " 1207	" "	" 19.
4. " 1202	" "	" 19.

Tested May 18.

	O.T. 1-20	Abortin 1-5
1.	+	++++ (slight necrosis)
2.	+	++++
3.	+	+++
4.	±	++

Since the O.T. in these tests had been recently made and had not been tested for potency, and the mild reactions with O.T. obtained above indicated a possibility of an insufficient dosage, these tests were repeated on the following day with an old lot of O.T. which had proved highly potent in 1-10 dilutions on many guinea pig tests.

relatively easy to sensitize to streptococci by the repeated intraperitoneal administration of dead or of living bacteria; that the skin reactions, best elicited with the Dick filtrates, become positive 2 to 4 weeks after the last injection; and that continued immunization causes this hypersensitiveness to fade. This point, again, is corroborative of Dochez and Sherman's observation on the neutralization *in vitro* of the allergic antigen by antistreptococcus serum, and of the observations of Mackenzie and Woo (13) on a similar fading of analogous sensitiveness induced with pneumococcus extracts.

In later work we extended our observations to allergy induced with a number of bacteria other than the tubercle bacillus, including the pneumococcus, staphylococcus, typhoid bacillus and *B. abortus bovis*. With all of these bacteria it was possible to render animals allergic in the same manner in which this was accomplished with streptococci. In considering the mechanism of these reactions, we were led to undertake investigations concerning the differences in the types of antibodies developed in animals by the injection of the several fractions of the bacterial cell, the results of which we published (14) simultaneously with similar studies by Avery and Heidelberger (15).

This work revealed relations which could not fail to have important bearing upon allergic reactions, if these were in any way dependent upon antigen-antibody combinations. Summarized briefly, it was shown that there was a fundamental difference in the nature of antibody production determined by the form in which the bacterial antigen was injected and upon the particular fraction of the bacterial extracts employed. To illustrate with the pneumococcus, which is the easiest organism to work with in this respect, the facts are as follows:

If a rabbit is immunized with whole pneumococci, the antibodies formed react with whole bacteria, agglutinating them in the usual way, and the same serum will precipitate the carbohydrate fraction or residue (soluble substance of Avery and Heidelberger). Since it is practically impossible to prevent some pneumococci from going into solution, even when formalin is applied directly to the young growths, such serum will also react with nucleoprotein.

If a rabbit is immunized either with pneumococci dissolved in bile and filtered,

Method of guinea pig preparation	Tested with abortin	Tested with staphylococcin
1. Dead <i>B. abortus</i>	Slight reaction	0
2. " " "	Large, hemorrhagic, severe	0
3. " " "	" not necrotic	0
4. " " "	Definite, not severe	0
5. Dead <i>Staphylococcus pyogenes aureus</i>	1 cm., redness, not raised	Slightly less than the abortin reaction on same pig
6. Dead <i>Staphylococcus pyogenes aureus</i>	0	0

Overlapping is further illustrated by a typhoid animal which had received 4 injections of formalinized typhoid bacilli intraperitoneally and 5 weeks after the last injection gave about equal reactions to typhoidin and 1-10 O.T. dilution, when before this it had given merely a weak typhoidin reaction and a negligible O.T. reaction.

It will be seen from these experiments, therefore, that there is a well defined specificity in bacterial allergy sufficiently definite to suggest an antigen-antibody mechanism; but that at the same time there is also a considerable amount of overlapping in animals that are highly sensitive, an overlapping not very different from that encountered in precipitation reactions with various types of bacterial nucleoproteins.

Further examples of specificity will be found recorded below in connection with skin tests on pneumococcus-sensitive animals.

III.

In preceding papers (10, 11) we have reported upon the production of allergic skin reactivity in guinea pigs treated with the formed cells and cell extracts of typhoid bacilli, staphylococci and streptococci. In all of these experiments the skin reactions obtained were of the delayed variety, appearing from 12 to 24 hours after intracutaneous injection, and manifested by well outlined reddened areas, slightly edematous and swollen—never, however, even in the most marked cases, showing the central necrosis and hemorrhage characterizing the most severe forms of tuberculin and abortin reactions.

Our experiments with Grinnell upon streptococci which, in harmony with the observations of Dochez and Sherman (12), showed that it is

The intracutaneous injection of formed precipitates resulting from the incubation of mixtures of residue and antiserum never produced typical skin reactions. The supernatant fluids of such mixtures were likewise negative.

From many experiments of this nature, together with the corroborative evidence of our other work, we feel confident in asserting that the reaction which is represented by the specific union of the type-specific residue or soluble specific substance of the bacteria (the haptophore group of the bacterial antigen) and their homologous antibodies (representing the agglutinins, precipitins, etc., of anti-bacterial sera) has absolutely no relationship to that form of bacterial allergy which is manifested by the delayed skin reaction. This, incidentally, is consistent with the results of Mackenzie and Woo, who found that no relationship existed between allergy and that protective mechanism which is represented by such antibodies.

The Relationship of the Bacterial Nucleoprotein and Its Antibodies to Allergic Reactions.

Having failed to demonstrate any relationship between the isolated residue antigen and the bacterial antibodies to the allergic reaction, we next proceeded to carry out skin reactions with bacterial substances so prepared that either the total suspended materials or their nucleoprotein constituents were represented in the test material. The test on page 763 is an experiment of this kind with pneumococcus.

If the following protocol is examined from the point of view of possible relationship between allergy and antinucleoprotein antibodies, it will be seen that skin reactions were obtained with bile solutions of the pneumococcus in animals in which residue reactions were negative, and that such reactions were best developed in animals treated with these same bile solutions. It stands to reason that the cited experiments represent only a few of a considerably larger number and the results such as those reported tend to indicate a possible relationship between nucleoprotein and its antibodies in the allergic phenomena.

or with the so called "nucleoprotein fraction," the antibodies formed react with such nucleoprotein, but hardly at all with the residue material mentioned above, and contain little or none of the ordinary antibodies that act upon the whole formed cells. Immunization with the residue fraction alone of course produces no antibodies whatever. The ordinary antipneumococcus sera put out for therapeutic and diagnostic purposes contain antibodies for both types. This must be borne in mind in subsequent considerations.

In taking account of those relations, then, it became necessary to sensitize in such a manner that both types of antibody would be likely to be formed and to do subsequent tests upon the skin with the materials specifically reacting with the respective antibodies. The pneumococcus offered the most convenient material for this purpose, largely because it has been found possible to destroy the complex antigen responsible for the formation of the anti-residue (anti-"whole" pneumococcus) antibodies by dissolving the bacteria in bile.

We proceeded first, therefore, to endeavour to define the relations of the carbohydrate residue antigen to the allergic reaction, as follows:

Active and Passive Sensitization to the Carbohydrate Residue (Soluble Specific Substance).

Our attempts to produce typical delayed skin reactions in animals with the carbohydrate, type-specific residue antigens were so uniformly negative that they may be very briefly summarized.

Whatever the method of active sensitization, subsequent skin tests with the homologous type-specific carbohydrate residue never produced typical delayed allergic reactions. Occasionally the residue injection gave rise to moderate immediate edematous swellings which disappeared within a short time and were usually gone completely on the following day, when typical reactions are most pronounced.

The same is true of animals treated with homologous sera potent with antibodies which specifically precipitated the residue *in vitro*. A great many attempts were made to sensitize passively in this manner and large amounts of serum were given in single and multiple injections; reactions were attempted at intervals ranging from a few hours after the injection of the serum up to several weeks. But the results remained negative and as described above.

An experiment with tubercle bacillus nucleoprotein is illustrated in the following:

The nucleoprotein extract was filtered through Berkefeld candles in order to remove possibility of injecting dead bacilli, or fragments.

The injections were made as follows:

Guinea Pig 1 received 6 injections ranging from 1 to 5 cc. at 4 day intervals between Jan. 13 and Feb. 3.

Guinea Pig 2 received 5 similar injections ranging from 5 to 7 cc. between Jan. 21 and Feb. 7.

Intracutaneous tests with nucleoprotein solutions and O.T. 1-5 were begun on these animals on Feb. 5 and carried out as follows:

Guinea pig	Feb. 5		Feb. 8		Feb. 10		Feb. 18		Feb. 19		Feb. 25	
	O.T.	N.P.	O.T.	N.P.	O.T.	N.P.	O.T.	N.P.	O.T.	N.P.	O.T.	N.P.
1	—	—	—	—	+	—	++	Not	++	+	—	—
2	—	—	±	—	±	±	++	done	+	+	+	+

Reactions in such an experiment are always better with O.T. than with pure nucleoprotein, a fact which we attribute to a possible denaturization of the nucleoprotein in production, since not only in these experiments but in all experiments such relatively purified nucleoprotein is a very poor antigen and must be injected in large quantities and in many doses.

In all experiments of this nature there is an eventual tendency to desensitization, that is, a fading of the reactions upon continuous treatment, an observation which is in agreement with those of Mackenzie and Woo. Apart from the practical significance of this, it is an immunological fact which tends further to arouse suspicion that such sensitizations may, to some extent, be dependent upon an antigen-antibody mechanism.

The fact that guinea pigs sensitized with tubercle bacillus nucleoprotein reacted well with O.T. suggested another attempt to sensitize with O.T. direct. This has not been noticed either by others or ourselves in the past as a result of repeated skin reaction, but when, in analogy with nucleoprotein sensitization, we injected guinea pigs with relatively large amounts of O.T.—that is, 3 to 6 injections of about 0.3 cc. of concentrated O.T. in proper dilution, intraperitoneally,

Guinea Pig 1 (845 gm.). Received 2 cc. of filtered bile solution of pneumococcus
 Guinea Pig 2 (670 gm.). on Mar. 23, 24, 25, 26 and 27, intraperitoneally.

Guinea Pig 3 (670 gm.). Received 2 cc. of 70°C. killed fresh suspensions of whole
 Guinea Pig 4 (620 gm.). pneumococci on Mar. 23, 24, 25, 26 and 27. Considerable loss of weight.

Intracutaneous Tests.

	Apr. 6		Apr. 10	Apr. 12		Apr. 13		Apr. 20	
	Skin test with bile solution	Skin test with whole pneumococci		Residue	Bile pneumococci	Pure bile 1-10*	Bile pneumococci	Residue	Bile pneumococci
1. Treated with bile solution	±	+	Reinjected intra-peritoneally with 2 cc. bile solution	-†	+++	±	+++	-	-
2. Treated with bile solution	+++	-		-	+++	±	++	-	++
3. Treated with whole pneumococci	+	-	Reinjected with whole, dead pneumococci	-	+	-	+	-	-
4. Treated with whole pneumococci	±	-		-	+	±	++	-	-

* These tests are a few of many that have been done with each preparation to check up the non-toxic action of remnants of bile. Our bile solutions of pneumococcus were all made by adding a minimum amount of ox bile for complete solution which ranged from concentrations of 1-15 to 1-10 with the suspensions used. It was impossible to separate the bile from the nucleoprotein by precipitation because such precipitation brings down the bile salts. These were gotten rid of in most of the preparations used, if not completely at least to a large extent, by 48 hours' dialyzing against salt solution.

† These residue reactions check up information previously obtained that the residue antigen gives no skin reaction in guinea pigs that are sensitized to the nucleoprotein, as shown by the bile solution reaction obtained on the same day. Again, there is no residue skin reaction in Animals 3 and 4, although, being treated with whole pneumococci, there must have been a formation of antibodies capable of reacting with the residue.

while the facts of active sensitization, specificity and desensitization, can be explained only on the basis of some mechanism analogous to that of antibodies, the passive experiment remains unconvincing. This may be interpreted as meaning either that we are dealing with a type of reaction in which antibodies, in the ordinary sense of the word, play no rôle whatever; or that such antibodies are indeed significant but represent only that part of the mechanism which determines specificity and desensitization, another additional factor being required to complete the reaction. It is this latter interpretation which seems to us the more likely one for reasons that will be elucidated in the following section in which we deal with the tuberculin reaction itself.¹

IV.

The Tuberculin Reaction Itself.

The preceding experiments may be regarded purely as preliminary to a study of the tuberculin reaction itself in that we believe that they have completely eliminated the possibility of a residue antibody mechanism in the tuberculin reaction, but have shown that a certain amount of sensitization can be obtained by active treatment with nucleoproteins. They have also again emphasized the existence of a specific element in these reactions, facts which to some extent clear up the underbrush but still leave us in the dark concerning the complete mechanism of the reactions.

Another series of preliminary experiments carried out during the past year too voluminous to be reported in detail, but necessary because of the many contradictions in the literature, may be briefly summarized as yielding the following information: (1) that contact *in vitro* of O.T. with the serum of animals immunized with living or dead tubercle bacilli does not produce anything which will give tuberculin

¹ We have so far failed also in obtaining any *in vitro* or *in vivo* neutralization of the allergic substance either by mixing with various antisera and incubating or by preceding skin tests by intravenous serum injections. To this we attribute less importance, however, since we bear in mind the great difficulty that Weil (16) and others have experienced in similar attempts with protein anaphylaxis where the antigen-antibody relations are far more clear. It seems that in all forms of sensitization it is not easy to divert antigen from sensitive cells, even with high concentrations of circulating antibodies.

filtered through Berkefeld filters to prevent the introduction of bacilli—we found that a sensitization could be obtained which was analogous to that with nucleoprotein; indeed, the animals reacted with large, flat, reddened areas, usually of considerably greater dimensions than those sensitized with nucleoprotein.

Experiments on Passive Sensitization to the Bacterial Nucleoprotein Fraction.

We will not go into detail concerning these experiments because the technique is simple and the results inconclusive. However, when a rabbit was treated with filtered nucleoproteins from the tubercle bacillus for a long time and with large quantities and the serum so obtained was found to precipitate tubercle bacillus nucleoprotein, normal guinea pigs were intraperitoneally injected with amounts ranging from 5 to 8 cc. and skin tests performed on them both with O.T. and nucleoprotein solutions every other day for some time after the serum administration.

It was found that in many of these pigs a definite allergic response was obtained both to O.T. 1-10 and to nucleoprotein solutions. These reactions did not, however, appear sooner than 6, 7 or 8 days after the serum administration, and usually faded within 3 weeks.

Similar experiments were done rather more extensively with pneumococcus substances in which we injected guinea pigs not only with the sera of rabbits immunized with bile extracts, but with the ordinary antipneumococcus Type I therapeutic sera which possessed a not inconsiderable capacity for precipitating nucleoproteins and bile solutions.

We do not cite protocols of these experiments because, carefully analyzed, they did not furnish conclusive evidence of passive sensitization. Although indicating the likelihood of such a process in many instances the results were complicated by the occasional development of similar sensitiveness in control animals prepared with normal horse serum. Moreover the consistent lateness of the appearance of sensitiveness whenever it appeared, indicated that the mere introduction of the antibodies could not be regarded as explaining the phenomenon as a whole.

We are confronted with the curious contradiction, therefore, that

3. Exudates obtained from normal guinea pigs with broth gave a very slight and probably non-specific sensitization to O.T. in 11 days to 2 weeks.

These experiments confirmed McJunkin's original claims, but did not shed any particular light on the mechanism of the reaction. Indeed, while it seems, as McJunkin supposed in his first report, that we are confronted with an active sensitization of some kind, this is not at all certain.

We now proceeded, therefore, to investigate the possibility that in the reaction between the tuberculous tissues and products of the tubercle bacillus there might be formed a toxic substance responsible for the reaction. The hope of obtaining some light in this direction was encouraged by two observations. One of these was an observation made with pneumococcus at about the same time that Julianelle and Reimann (19) published their observations upon the purpura-producing, autolytic substance derived from pneumococci. The observation was as follows:

If a freshly prepared pneumococcus suspension is divided into three parts, one of them left undisturbed except for the addition of a minute amount of thymol, the second immediately heated to 65° for 15 minutes and the third dissolved in bile, a similar amount of thymol added to the last two to equalize conditions and all three set into the incubator and allowed to stay there for 48 to 72 hours, and skin reactions carried out with 0.2 cc. of each of these suspensions in a normal guinea pig, there will be very feeble, or practically no reactions over the bile solution area or that carried out with the pneumococci immediately heated, but over the area into which the autolyzed pneumococci were injected there will be formed a violent delayed reaction which in all its morphological and pathological features resembles a violent, severe tuberculin reaction in a tuberculous guinea pig injected with O.T. These relations are shown in the accompanying figure.

Similar experiments carried out with meningococcus show comparable conditions, except that in meningococcus the immediately heated organisms often showed a reaction similar to but milder than that of the autolyzed ones.

With *Bacillus abortus bovis* an analogous experiment can be performed, except that here autolysis seems to be extremely slow and reactions comparable to the allergic ones do not develop until the unheated cultures have been kept in the incubator for 8 or 9 days and then they are not as violent as those produced with pneumococcus.

reactions in the normal animal, whether or not complement be present; (2) that the same is true when the serum of animals immunized with tubercle bacillus nucleoprotein is employed; (3) that precipitates formed in both types of reaction fail to produce tuberculin reactions in normal guinea pigs, even when treated with complement; and (4) that none of the sera mentioned above will neutralize the action of O.T. upon tuberculous guinea pigs.

These facts in general correspond quite closely with those ascertained for bacterial allergy with other organisms. A direction of experimentation which was now indicated was that which dealt particularly with the meaning of the inflammatory tissue reactions, the tubercles, which may be recognized as possessing considerable significance in the development of tuberculin hypersensitiveness. In a former paper we have already shown that a certain amount of passive sensitization can be obtained by the injection into guinea pigs of sera from rabbits in which a large amount of tuberculous inflammatory reaction has been incited by the establishment of multiple tubercles. We were also able at that time to confirm the observation of Lange (17) that some sensitization can be incited by repeated injection of tuberculous tissue filtrates. In none of this work, however, did the sensitization amount to very much more than that which we have more recently obtained with nucleoproteins. Never was there any indication of hemorrhage or necrosis. These experiments have been repeated with the same results.

We therefore investigated the McJunkin (18) experiment in an effort to procure from it a possible clue, since we have been able in the past to confirm this procedure by the method originally reported by him. In attempting to analyze this method we determined the following facts:

1. A normal guinea pig injected with a filtered exudate from the peritoneum of a tuberculous guinea pig killed by intraperitoneal injection of O.T., gives an excellent skin reaction 11 to 12 days after the administration of the filtrate.

2. Similar exudates obtained by injecting a tuberculous guinea pig with broth sensitized definitely but less strongly than in the first case, also in about 11 days.

satisfied that we were obtaining anything more than definite suggestions in a positive direction until we abandoned the efforts with tissue extracts and began to work with macerated cellular materials, unfiltered. The observation which most encouraged us to continue in this direction was the fact that we again and again noticed that mixtures of O.T. with tissue extracts, even though they had relatively little action upon a normal animal, were considerably more toxic than O.T. alone for the tuberculous animal.

We do not cite a large number of unsuccessful experiments, but restrict ourselves to typical examples of the final experiments which convinced us that the interaction between O.T. and tuberculous tissue played an important rôle in the mechanism of tuberculin reactions. The following is such an example.

Mixtures were made as follows: Lung tissue of a tuberculous guinea pig was macerated in a mortar with sterile sand, and small amounts of salt solution added. A similar maceration of normal lung was made at the same time. The following preparations were then set up in test-tubes.

1. Macerated bits of lung in salt solution, 5 cc., + 0.1 cc. of concentrated O.T.
2. Similar tuberculous lung without O.T.
3. Salt solution, 5 cc., + concentrated tuberculin, 0.1 cc.
4. Normal lung, 5 cc., + O.T., 0.1 cc.

To each one of these tubes a small bit of thymol was added in order to prevent putrefaction, and the tubes were incubated. After 24 hours small amounts of fluid were taken from each of these tubes, centrifuged at high speed and 0.2 cc. respectively injected into a large white normal guinea pig.

The results are shown in Fig. 5 and are self-explanatory. It will be seen that while the normal lung macerate and the O.T. alone produced slight reactions, the incubated tuberculous lung macerate with O.T. gave a strong and extensive reaction.

Another experiment of the same type done with human lung is the following:

Normal human lung and lung thickly studded with miliary tubercles were obtained through the kindness of our hospital associates. Pieces of each were cut up with a pair of scissors and macerated with sand in a mortar, salt solution being added. Finally the sand was allowed to settle and the supernatant suspension containing bits of lung was taken up into tubes as follows:

1. Normal human lung suspension, 10 cc.
2. Normal human lung suspension, 10 cc., + O.T., 0.3 cc.
3. Tuberculous lung suspension, 10 cc.
4. Tuberculous lung suspension, 10 cc., + O.T., 0.3 cc.

These experiments indicated the possibility that bacterial products may yield, upon cleavage, a substance toxic for the normal animal as O.T. is for the tuberculous animal.²

It was obviously suggested, therefore, again to investigate the results of the incubation of mixtures of O.T. with the extracts of tuberculous tissues.

This thought was further encouraged by observations that had been made in connection with earlier attempts to neutralize O.T. for tuberculous animals by similar incubation. An experiment of this type is as follows:

A mixture was made of one part of O.T. with five parts of a clear, filtered extract made by macerating and shaking the skin of a tuberculous animal in slightly alkaline salt solution. The mixture was allowed to stand for several hours in an incubator and overnight in the ice box.

0.1 cc. of this was then injected into a tuberculous animal, and in another spot a fresh 1-5 solution of the same tuberculin in salt solution. As shown in the figure, in this particular experiment the reaction over the point of injection of the mixture was markedly larger than that which developed where the fresh O.T. dilution had been injected.

Conditions similar to the above were encountered on a number of occasions, usually when extracts of lung or skin were used, less frequently when the tuberculous tissue extract was furnished by spleen or liver tissue.

From these two types of experiment it seems quite likely that in addition to the ordinary antigen-antibody reaction, tuberculin allergy was in some way related to a direct reaction between the inflammatory tissue reactions and the tuberculin, a thought which, incidentally, is suggested by all the past history of studies of tuberculin reactions.

Experiments on the Action of Tuberculous Tissue upon O.T.

This line of investigation was followed for a very long time with tantalizingly encouraging, but inconclusive results. We were never

² We assume that this toxic substance is the same as the purpuric poison described by Julianelle and Reimann, since it is produced in more or less the same way and since it possesses a considerable heat stability. The powerful toxicity of this material for guinea pigs, as shown in the figure, opens the question of its relationship to other described pneumococcus poisons and necessitates the further investigation of its possible antigenic properties, a matter that is being worked upon.

Sensitiveness develops usually within 10 days after the first dose and increases with continued treatment for 3 or 4 weeks.

Sensitiveness is relatively specific, by which we mean that there is a definite specificity which, however, in highly sensitive animals is not absolute and shows considerable overlapping.

Continued treatment with considerable quantities of the above substances leads to gradual desensitization in animals in which there are no chronic foci present, which, as in tuberculosis, tends to continue the sensitization.

Attempts at passive sensitization have been irregular and inconclusive. When any degree of sensitiveness has developed after the injection of immune sera, it has appeared late and has been of doubtful specificity. Conversely we have failed in any case to neutralize the activity of the active allergic constituents of bacterial extracts by incubation with any type of immune serum.

We have failed so far to show any increased fixation of tuberculin material on the part of tuberculous tissues or on that of living tuberculous animals. These failures, however, seem to us of relatively slight importance since quantitative experiments of this nature are extremely difficult in the case of a substance as delicately potent for the tuberculous animal.

On the other hand we have obtained definite, though irregular evidence that the incubation of O.T. with fragments of tuberculous lung tissue (less clearly with other tissues) leads to the formation of a substance that produces allergy-like lesions in the skin of normal guinea pigs. With somewhat greater regularity, similar treatment of O.T. has enhanced the potency of the tuberculin for tuberculous animals. And, in these experiments there was evidence that the factor responsible for this action was not easily separable from the cells themselves.³

When these experimental data are analytically considered they appear in many respects confusing and contradictory. There has been so much work done on the tuberculin reaction, moreover, that, in the face of experimental inconsistencies it would seem foolhardy to formulate more than tentative suggestions to explain the mechanism

³ We are permitted by Dr. Petroff of Saranac to state that he has obtained results similar in principle with our own, but also irregular and not repeatable at will

Small, approximately equal pieces of thymol were added to each tube. After 48 hours' incubation portions of each tube were taken out, centrifugalized at high speed and 0.2 cc. injected intracutaneously into a normal, large, white guinea pig.

The results are shown in Fig. 6. Again the area into which supernatant fluid from Tube 4, containing the tuberculous lung suspension with O.T., had been injected gave a reaction larger than that given by the material from Tube 3, and both of these were much more extensive than those from the two controls.

We assume that the results in Tube 3 of both experiments were due to the presence of a certain amount of tubercle bacillus material in the infected tissue.

SUMMARY.

Our experiments have confirmed the fact that the so called bacterial allergies are dependent upon a mechanism which differs materially from that determining true protein anaphylaxis. Anaphylaxis to protein substances of the bacteria probably occurs but plays a relatively unimportant rôle in the phenomena of infection. The bacterial allergies, however, are of great importance since they develop rapidly and render the infected animal highly vulnerable to products of the bacterial growth which are relatively innocuous for the normal animal.

Neither the type-specific carbohydrate "residue antigens" (the "soluble specific substances" of Avery and Heidelberger) nor the antibodies reacting with them play any part whatever in bacterial allergy, and since these type-specific substances represent the haptophore groups of the whole bacteria by which they react with the agglutinins, precipitins, sensitizers, etc., of immune serum, allergy, as previously determined by Mackenzie and Woo, is in no way related to that phase of resistance which is determined by these antibodies. This does not, however, preclude the possibility that allergic hypersusceptibility may not in some way be related to other factors of resistance more definitely associated with cellular rather than with intravascular reactions. Our previous studies with Jennings and Ward in tuberculosis point in this direction (20).

Guinea pigs can be actively sensitized with all the bacteria with which we have worked when repeated injections of whole bacteria or of the protein (nucleoprotein) fraction are administered. Large amounts of the latter are necessary since these materials are indifferent antigens, possibly because of the severe manipulations necessary in their production.

The process of allergy, as far as we can approach it then, may be conceived as follows:

A nitrogenous, probably protein, constituent of the bacterial growth or of its body substance stimulates a specific reaction in the tissue cell by which its specific capacity to establish contact with this constituent is enhanced.

The cell is thereby enabled to exert a, probably, enzyme-like effect upon this material in consequence of which a toxic substance is liberated, largely upon or possibly within the cell itself.

Both processes may be dependent upon one and the same reaction body. But it seems more likely that increased contact and the increased cell activity are separately developed, an assumption which is rendered probable by the association of the highest degrees of allergy with inflammatory cell reactions, and by the fact that moderate and less specific allergic sensitiveness follows 10 or more days after the administration of considerable amounts of indifferent protein substances to guinea pigs. We interpret this as signifying that such injections may non-specifically increase cellular activity, a change which many earlier workers have spoken of as "cell irritability."

Both processes are closely associated with the altered cell itself and the factors by which the reaction is brought about are not easily given up to the blood stream as are the antibodies formed in response to injections of proteins or whole bacteria.

We are confronted, therefore, with an immunological mechanism which has some close analogies to those others in which circulating antibodies are formed, but which differs from these mainly in the intimacy with which the entire reacting system is associated with the cells themselves.

It is difficult to conceive that a functional cell alteration, as profound as this, should be entirely unrelated to the phenomena of susceptibility or resistance.

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of these reactions. Nevertheless there are a few outstanding and sufficiently reliable facts which compel a limited number of definite deductions.

In the first place there is no question of the complete independence of the true allergic phenomena from the ordinary bacterial antigen-antibody reactions. We know, moreover, that the allergic substance is chemically separable from the carbohydrate "residue" or haptophore group of the bacteria (Mueller, Laidlaw and Dudley). Indeed it has been shown by Long and Seibert (21) that the active allergic substance is either a protein in itself, or at any rate closely associated with the bacterial protein.

Furthermore, the distinct, though limited, specificity of the allergic sensitiveness compels the conclusion that we are dealing with an immunological process in which the tissue cells acquire an increased specific capacity to react with this nitrogenous material, a capacity which, in principle, is not far removed from the supposed "sessile receptor" apparatus which is conventionally held responsible for protein anaphylaxis; and this analogy is further amplified by the apparent desensitization which continued treatment produced in many of our own experiments as well as in those of Mackenzie and Woo.

Here, however, the analogy with protein anaphylaxis ends. Passive sensitization with any form of immune serum or with the sera of highly sensitized animals is either feeble or entirely unsuccessful and indicates quite convincingly that, whatever the receptor apparatus of the cells may be, it is not easily given up to the blood stream as are ordinary antibodies. Further than this, our tissue-tuberculin experiments, irregular and occasional as they were, nevertheless convinced us that:

1. The contact with the tissues of tuberculous animals results in the production of a toxic factor, not unlike the autolytic toxic materials of some bacteria.

2. The active cell constituent by which this action is wrought, is not easily separated from the cells, even by energetic methods of extraction.

This close association of the entire process with the cells themselves is particularly significant in view of the obvious cell injury in which these delayed allergic effects differ from the ordinary urticarial, evanescent reactions associated with protein anaphylaxis.

4. Tuberculous human lung fragments + O.T. in a concentration similar to the above.

Small pieces of thymol had been added to all the preparations to prevent infection with bacteria.

Small amounts of fluid were removed with a pipette and centrifuged at high speed, the clear supernatant fluid being used for intracutaneous injection.

FIG. 6. Comparative areas of inflammatory edema on the skin of a normal guinea pig resulting from intracutaneous injections with the following materials:

1. Normal lung fragments + O.T., total concentration 1-10.
2. O.T. 1-10 in salt solution.
3. Tuberculous lung without O.T.
4. Tuberculous lung with O.T.

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EXPLANATION OF PLATES.

PLATE 29.

FIG. 1. Severe type of skin reaction given in guinea pigs sensitized by active immunization with nucleoprotein. This particular guinea pig happens to have been one sensitized by the McJunkin method with the exudate produced in a tuberculous guinea pig with O.T., but the type of the reaction represents the average severity of the non-necrotic delayed skin reactions referred to in the text.

FIG. 2. Comparative reactions in an *abortus*-sensitized pig during the early stages of severe reaction with (1) 1-10 tuberculin and (2) 1-5 abortin.

FIG. 3. Typical severe skin reaction on a normal guinea pig obtained by the injection of 0.2 cc. of pneumococci autolyzed in salt solution for 48 hours.

PLATE 30.

FIG. 4. Tuberculous guinea pig tested over Area 1 with O.T. 1-5, over Area 2 with O.T. diluted to the same extent with concentrated extract of the skin of a tuberculous guinea pig and kept in the incubator for 4 hours before the two tests were done. Note the much larger extent of the area over 2.

FIG. 5. The figures in this illustration show the resulting inflammatory areas on the skin of a normal guinea pig injected intracutaneously as follows:

1. Supernatant fluid from a 2 days' incubation of fragments of normal lung tissue with O.T. in a concentration of 1-33.
2. O.T. in solution 1-33, similarly incubated.
3. Tuberculous human lung fragments in salt solution.

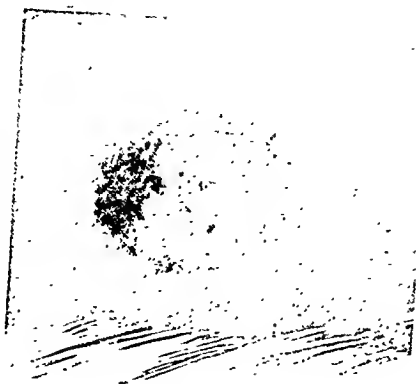


FIG. 1.



FIG. 2.



FIG. 3.

(Zinsser and Tamiya: Analysis of bacterial allergy.)



FIG. 4.



FIG. 5.



FIG. 6.

(Zinsser and Tamiya: Analysis of bacterial allergy.)

Occurrence in Filtrates.

Several tubes of cooked meat were inoculated and allowed to incubate for 5 days at 37°C. Part of the tissue having liquefied, the supernatant fluid was removed and passed through a Berkefeld filter. The filtrate was incubated along with subcultures from it. After 2 weeks both were found sterile.

The level of the meat in three tubes of cooked meat medium was carefully marked as above. The first tube received 1 cc. of the sterile filtrate. The second received 0.5 cc. and the third tube was not opened. All three were incubated at 37°C. After 18 hours the meat level in the two tubes containing the filtrate was about 2 mm. below the mark. The control tube was unchanged. After 5 days the meat level in the filtrate-containing tubes was about 8 mm. below the mark while the control was still unchanged. It appears that a true ecto-enzyme is responsible for the tissue digestion. The name histase is proposed for this enzyme.

Effect of Heat on the Enzyme.

The meat level in six tubes of cooked meat medium was carefully marked. To the first was added 1 cc. of the sterile filtrate described above. A tube containing 5 cc. of the filtrate was then immersed in a water bath held at 60°C. After waiting 4 minutes for the temperature to become uniform, 1 cc. of the filtrate was removed at 15 minute intervals and placed in one of the marked tubes of cooked meat. All tubes were then incubated at 37°C. with an unopened control and a sterility control. Tissue in all tubes containing filtrate was digested, but not at the same rate. After 5 days visible digestion had taken place at about equal rates in tubes heated not longer than 30 minutes. About 2 days later, liquefaction was observed in the 45 minute tube and about 3 days subsequent to this, digestion was discernible in the tube heated for 1 hour. After 2 weeks all tubes showed about the same degree of digestion. Exposure to 60°C. for 1 hour, therefore, seems to retard the action of the enzyme but not completely to destroy it. It may be that most of the enzyme was destroyed but that sufficient remained to digest a few mm. of the meat.

TISSUE-DIGESTING ENZYME (HISTASE) OF STREPTOCOCCI.

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In the fall of 1925, while making a study of beta type hemolytic streptococci from a variety of sources, a strain was isolated from impetigo-like lesions which was found to be capable of digesting a considerable portion of the meat in tubes of Holman's (1) modification of Robertson's cooked meat medium. It did not liquefy gelatin and in every other respect resembled *S. pyogenes*.

Digestion of animal tissues by streptococci of the type represented by *S. pyogenes* has not been described before, as far as can be ascertained from a search of the available literature. Digestion of casein has been observed and reference to this will be made later on.

Description of the Tissue Digestion.

Tubes of cooked meat medium with vaseline seals were inoculated with the proteolytic strain of streptococcus referred to above. The level of the chopped meat in the tubes was carefully marked with a red wax pencil. After 24 hours at 37°C. no change in the level of the meat had occurred. After 48 hours, however, the level slowly descended. Smears made at this time showed pure cultures of the streptococci. After 5 days at 37°C. about 30 per cent of the meat had completely disappeared. The meat continued to be liquefied during 3 subsequent weeks at room temperature. At the end of 1 month about 75 per cent of the meat had disappeared and the remainder had the finely granular appearance sometimes seen in cooked meat cultures of very proteolytic anaerobic bacilli. No gas or foul odor was produced.

end of 90 minutes. The results of the comparisons are shown in Table I. This series would seem to indicate that proteolysis and hemolysis by this type of organism are not closely related. It is

TABLE I.
Relation between Hemolysis and Proteolysis.

Strain No.	Source	Tube hemolysis* (90 min.)	Meat digestion
8556	Septicemia	4+	—
3606	Certified milk	—	—
Reed.	Infected ear	4+	—
8674	Septicemia	—	+
Mort. 23	Septic sore throat	4+	+
Lexley	Infected ear	4+	+
X 40	Septic sore throat	4+	+
8616	Erysipelas	4+	+
S 2	Scarlet fever	4+	+
X 32	Septic sore throat	2+	+
X 39	Septic sore throat	4+	—
X 41	Septic sore throat	4+	+
Cow 108	Mastitis	4+	+
3641	Certified milk	+	±
Hamer	Sore throat	4+	+
2735	Certified milk	4+	+
8576	Septicemia	+	±
2082	Certified milk	4+	+
3639	Certified milk	+	—
2081	Certified milk	4+	—
Hebron	Sore throat	4+	+
2600	Certified milk	4+	—

* 4+ indicates complete hemolysis.

Summary:

Strains digesting meat and producing soluble hemolysin.....	12
Strains digesting meat and not producing soluble hemolysin.....	1
Strains not digesting meat but producing soluble hemolysin.....	6
Strains not digesting meat and not producing soluble hemolysin. . .	1
Doubtful.....	2

possible that the method used for determination of hemolysin production is too crude. De Kruif and Ireland (3) have found that hemoly-

Action of the Enzyme on Human Tissues.

In preparing the cooked meat medium referred to above, the method of Holman (1) was adopted, with the exception that beef hearts were used and vaseline seals were added to the tubes. Similar tubes were prepared with human instead of bovine heart. The human heart was taken from an uninfected case at autopsy. Nine strains of hemolytic streptococci were studied in this medium. Of these, three did not digest bovine or human heart, while six digested both.

In several tubes inoculated with streptococci and sealed with vaseline, digestion failed to occur. Smears were made to determine whether growth had occurred. It was found to be so sparse as to leave the ability of the organisms to digest meat in doubt. The vaseline seals were removed with sterile pipettes and the cultures reincubated. After 24 hours good growth had occurred and after 5 days extensive digestion was observed. These strains were evidently fairly strict aerobes. This peculiarity has since been observed in several other cases. A difference in the appearance of the meat in tubes treated in this manner deserves mention. In such tubes, after growth has occurred, the color of the meat changes from the usual red-brown to a greenish grey most intense at the top surface. The meat in which growth has occurred without the removal of the vaseline seal does not change color, or at most becomes a slightly darker brown. Strains which digest meat in the presence of the vaseline seal, do not produce any color change when cultivated in tubes from which the seal has been removed, although they still digest the meat.

Relation to Hemolysin.

Julianelle (2), in a study of the hemolytic staphylococci, obtained data suggesting that proteolysis and hemolysis by those organisms are somewhat closely related. With this in mind twenty-two strains of hemolytic streptococci from various sources were tested for ability to digest meat and to produce hemolysin. For the hemolysin test the organisms were cultivated for 18 hours in infusion bouillon at 37°C. In each test 0.5 cc. of culture was mixed, with 0.5 cc. of 5 per cent suspension of washed rabbit corpuscles. The mixtures were shaken and placed in the water bath at 37°C. Readings were made at the

cultures above mentioned. The tests for casein digestion were at first made by inoculating tubes of brom-cresol purple milk. Most of these promptly coagulated, and it was difficult to distinguish between small amounts of digestion and separation of the whey. The method was abandoned.

Following the procedure of Eijkman (4), infusion agar was melted in tubes and to each tube, containing about 12 cc. of agar, was added 1 cc. of sterile milk. The mixtures were poured into plates and the surfaces were streaked with the streptococci to be tested. Hydrolysis of the casein is evidenced by a clear area about the colonies which fails to become clouded upon flooding the plate with 25 per cent acetic acid. The results of these tests are recorded in Table II.

From an inspection of this table it is seen that there is no relation between ability to digest tissue and liquefaction of gelatin. This of course applies to the method and strains used. Ability to digest bovine or human tissue does not imply ability to liquefy coagulated bovine serum. Ability to hydrolyze casein is not paralleled by ability to digest tissue, serum or gelatin. The enzyme which digests tissues appears therefore to be distinct from other proteolytic enzymes produced by streptococci.

Nature of the Enzyme.

Ten cultures of hemolytic streptococci in cooked meat medium, all of about the same age (1 month) were selected. Five of these showed considerable digestion. Two were doubtful, the meat level being only about 1 mm. below the line, while the remaining three were distinctly negative. Two were cultures which had shown proteolytic activity only after removal of the vaseline.

Being careful to avoid stirring up the meat, 1 cc. of the clear supernatant fluid was removed from each tube and formol titrations made by Brown's modification (14) of Sørensen's method. This modification is particularly adapted for use with bacteriological media. Table III shows the results of the titrations. It is to be seen in Table III that the cultures showing extensive digestion after 5 days growth at 37°C. also contain large amounts of substances determinable by the formol titration. The extent of the digestion roughly parallels the increase in such substances. The doubtful cultures as well as the

sin may disappear from cultures more than 14 hours old. It is evident however that some strains of streptococci may produce a large amount of hemolysin yet fail to digest meat.

TABLE II.

Comparison of the Proteolytic Activities of Various Species of Streptococci.

Strain No.	Source	Species*	Meat digestion†	Casein digestion‡	Liquefaction of gelatin‡	Digestion of blood serum
8616	Erysipelas	<i>Pyogenes</i>	+	+	—	—
X 40	Epidemic sore throat	<i>Pyogenes</i> §	+	—	—	—
3639	Certified milk	<i>Pyogenes</i>	—	+	—	—
8591	Bronchopneumonia	<i>Pyogenes</i>	—	—	—	—
3056	Certified milk	<i>Pyogenes</i>	+	—	—	—
2735	Certified milk	<i>Pyogenes</i>	+	—	—	—
2082	Certified milk	<i>Infrequens</i>	+	—	—	—
S 2	Scarlet fever	<i>Pyogenes</i>	+	—	—	—
Lexley	Infected ear	<i>Pyogenes</i>	+	+	—	—
Lanc.	Impetigo-like lesion	<i>Equi</i>	—	—	—	—
8674	Septicemia	<i>Pyogenes</i>	+	+	—	—
3636	Certified milk		—	+	—	—
3052	Certified milk	<i>Infrequens</i>	+	—	—	—
8556	Postabortion septicemia	<i>Infrequens</i>	—	—	—	—
8741	Bronchopneumonia	<i>Pyogenes</i>	+	+	—	—
8576	Septicemia	<i>Pyogenes</i>	—	—	—	—
Hamer	Sore throat	<i>Pyogenes</i>	+	+	—	—

* Holman's classification (12).

† 7 days at 37°C.

‡ 48 hours at 37°C.

§ *S. epidemicus* of Davis (13).

Relation of Tissue Digestion to Liquefaction of Gelatin, Coagulated Blood Serum and Casein.

Eighteen strains of hemolytic streptococci were tested for ability to digest meat, casein, coagulated blood serum and gelatin. To determine gelatin liquefaction, tubes of infusion gelatin were melted in the incubator. These were inoculated with 1 drop of 24 hour bouillon cultures. The coagulated serum was prepared in the form of Loeffler's slants. These were inoculated with 1 drop of the bouillon

strictly anaerobic streptococci which are capable of breaking down tissue proteins. These organisms produce gas and foul odor and the process appears to be quite unlike that described in this paper. MacCallum and Hastings (8) described a type of proteolytic streptococcus which they named *M. zymogenes*. This organism was obtained from a case of acute endocarditis. It liquefied gelatin, digested coagulated blood serum and peptonized milk. The proteolytic enzymes were found to be active in sterile filtrates. This organism differed from the typical *S. pyogenes* in being practically always arranged in pairs, in its extremely small size and in being able to survive for 3 or 4 months at room temperature upon agar slants in spite of their dried condition. The appearance upon blood agar was not described.

The digestion of tissues, as described in this paper, by ordinary types of hemolytic streptococci seems to be a new observation. The value of such a readily demonstrable characteristic, when exhibited by a considerable percentage of strains lies in its possible use as a means of classification or identification. The tendency in the past has been to rely almost wholly upon the appearance upon blood agar and fermentation reactions. It is only comparatively lately that the use of other cultural characters has become extensive. Among these characters may be mentioned the hydrolysis of sodium hippurate (9) and growth at various surface tensions (10, 11). In view of the unsatisfactory situation existing in connection with identification and classification of the streptococci, it would seem desirable to make more extensive use of such simple tests as hydrolysis of casein, digestion of animal tissues, etc., for these purposes.

SUMMARY AND CONCLUSIONS.

1. An extracellular, proteolytic enzyme has been observed in more than 30 strains of beta type, aerobic and facultative hemolytic streptococci.
2. The enzyme is readily demonstrable in sterile filtrates of cooked meat cultures.
3. No gas or foul odor is produced.
4. It is partially inactivated by exposure to about 60°C. for 45 minutes or longer.

frankly negative ones show little increase in these bodies, the doubtfuls containing little more than the negatives. These results seem to show that the enzyme resembles trypsin in its action.

Further Studies.

To date about 58 strains of hemolytic streptococci have been studied. These represent a variety of sources, bovine, human and otherwise. At present it is sufficient to state that about 30 of them

TABLE III.

Formol Titration of the Supernatant Fluid in Tubes of Cooked Meat Medium Containing Cultures of Various Hemolytic Streptococci.

Strain No.	Approximate per cent of meat digested in 5 days at 37°C.	Quantity of N/20 NaOH needed for formol titration of 1 cc. of the culture
Sterile No. 1	—	0.25
Sterile No. 2	—	0.12
8616	30	4.23
3052*	15	0.55
8741	25	4.85
X 40	25	4.13
8576	57	0.47
8591	57	0.41
Lanc.	0	0.40
2082*	12	0.57
4d ign.	0	0.31
S. H.	0	0.40

* Digested meat only after removal of the vaseline seal.

digest meat vigorously. There seems to be no relation between this power and source, pathogenicity or fermentative reactions. A more detailed report of this will be made later.

DISCUSSION.

Proteolysis by streptococci has been observed before. Casease has been frequently described both in culture filtrates and in extracts of washed cells (5, 6). The presence of peptase is well known to be of common occurrence. Prevot (7) has described certain types of

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5. The enzyme manifests itself in cooked meat cultures after about 48 hours incubation at 37°C. The sterile filtrate from a 10 day old culture acts within 18 hours.

6. From 50 to 75 per cent of the meat in a tube of cooked meat medium may be digested in about 3 weeks at room temperature after 5 days initial growth at 37°C.

7. No correlation is found, in the cases studied, between hemolysis and proteolysis.

8. Streptococci not digesting beef tissue will not digest human tissue, and those which do digest beef tissue also digest human tissue. This conclusion applies only to the nine strains studied.

9. Ability to digest animal tissues does not necessarily imply ability to digest casein, coagulated beef serum or gelatin.

10. The disappearance of the meat from cooked meat cultures of hemolytic streptococci is quantitatively roughly paralleled by increase of formol-titrable substances in the fluid portion of the medium.

11. The enzyme resembles trypsin in its action. Streptococci from a variety of sources, bovine, human and otherwise have shown varying degrees of proteolytic activity.

12. The name histase is proposed for this enzyme.

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rather than bacilloid. Detweiler and Hodge (8) in Toronto grew 3 strains morphologically similar to *Bact. pneumosintes* from filtered influenza material, 2 from lung filtrates of injected animals, 1 from filtered nasopharyngeal washings. Subcultures failed to grow, so identification was not completed. Finally Thomson (9) isolated a minute organism from a case of Engadine fever—a type of influenza endemic in Switzerland—and obtained 2 other strains from influenza patients in England.¹ In all of these studies control experiments with non-influenzal material have been uniformly negative.

Thus the presence of an anaerobic, Gram-negative, filter-passing bacterium, identified as *Bact. pneumosintes*, in the human respiratory tract only in the early hours of an influenzal infection, has been established in many parts of the world. This in itself is but a beginning, however, in determining the relationship of *Bact. pneumosintes* to the clinical disease. A large accumulation of observational and experimental evidence must be sought wherever available, and pieced together as opportunities permit.

The Outbreak of 1926.

When we undertook an investigation of the presence of *Bact. pneumosintes* in clinical influenza in New York City last March (1926), the brief local outbreak proved to be already on the wane. Consequently we had an opportunity to see only a few cases. It was reported that clinically the infections varied considerably in mode of onset, relative prominence of various signs and symptoms, and the blood picture. Yet they would be grouped together with the common designation "influenza" as typified by sudden onset, sometimes with chill, a sharp fever and marked prostration, headache and other pains, absence of profuse coryza, and prolonged depression during convalescence. Sometimes family infections, and more often isolated

¹ While this paper was in press M. W. Hall (*J. Exp. Med.*, 1926, xliv, 539) reported the experimental production of characteristic lung lesions in rabbits and guinea pigs with nasopharyngeal washings from a patient with typical epidemic influenza. From one of his affected animals a culture of *Bact. pneumosintes* was obtained. The presence of this organism in the lungs of experimentally infected animals predisposed them to the pulmonary localization of other bacteria and the production of definite secondary pneumonic lesions.

BACTERIUM PNEUMOSINTES IN CLINICAL INFLUENZA IN NEW YORK CITY IN 1926.

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INTRODUCTION.

Since the first paper in the series by Olitsky and Gates (1) in which the presence of minute filter-passing bodies (*Bacterium pneumosintes*) in cultures from affected rabbits' lungs and from filtered nasopharyngeal washings of influenza patients was reported, a number of investigators in widely separated laboratories have described the isolation, under similar conditions, of anaerobic filter-passing organisms, identified morphologically and sometimes serologically as *Bact. pneumosintes*. Such organisms were "obtained consistently" by Loewe and Zeman (2) in New York, from the filtered nasopharyngeal washings of patients with epidemic influenza, and produced a characteristic clinical and pathological picture (3) when injected into experimental animals. Then Gordon (4) in London reported evidence of the same bacterium in 14 of 20 influenza cases, and filtrates of the bronchial secretion in 3 fatal cases yielded 2 cultures. In 1922 also, Lister (5), in South Africa, obtained 5 cultures of an identical anaerobe in 11 instances in which influenzal washings were cultured within 24 hours of the acute onset, and reported 4 febrile reactions, 1 fever and drop in the leucocyte count, and 1 case of typical influenza among 12 volunteers sprayed with unheated cultures of the filter-passing organism. No reactions occurred among 6 volunteers sprayed with heated cultures. In 1923 Nakajima (6) in Tokio cultivated 2 strains from pharyngeal washings and 1 from the lung tissues of a fatal case of influenza. Seitz (7) in Zurich observed masses of very tiny bodies in the respiratory exudates of influenza patients and grew them for a time in mixed sputum cultures. He regards these bodies as coccoid

rabbits, representing 6 patients, showed significant reactions, such as fever, a drop in the leucocyte, and especially the monocyte, count, and typical gross and petechial hemorrhages in large, edematous lungs. 6 rabbits injected with whole or filtered lung tissue from 4 of these animals showed similar but less striking effects in the 2nd passage. A 2nd passage apparently failed in 4 other transfers from 2 of the other 5 rabbits.

This series of rabbit injections was carried out under a serious handicap. Our stock of rabbits was low, and the immediate demands of the situation required the use of untested animals. Although only apparently healthy rabbits were chosen for injection, the whole lung tissues of most of them subsequently showed infection with *B. lepi-septicus* or *B. bronchisepticus* and cultures of them had to be discarded. In the presence of these concurrent infections a strict interpretation of the reactions of most of these rabbits is not justified. The series was therefore discontinued and our attention turned to the direct cultivation of filter-passing, anaerobic organisms from the nasopharyngeal washings, and from filtered material from the rabbits' lungs.

Cultivation Experiments.

Having in mind that often very sparse growths of filter-passing anaerobes are obtained in early generations, and might easily be missed in the Smith-Noguchi medium, we made at least 2 successive transfers of every primary tube that showed no growth when subplanted on aerobic blood agar plates. The control tubes set up to test the sterility of our media were likewise transferred, to avoid the possibility of false evidence from an extraneous source. Material from each generation was also examined microscopically in stained smears.

This tedious procedure proved to be justified in 6 series of cultures in which minute anaerobic organisms were obtained. 2 of these have been definitely identified as strains of *Bact. pneumosintes*. In the first 2 generations of these *pneumosintes* cultures the growth was so sparse as to escape microscopic detection and no visible colonies developed in subplants on anaerobic blood agar plates. But in the 3rd, 4th, and subsequent generations the typical clouding of the Smith-

cases occurred. Compared with the influenza epidemic and its repercussions of 1918 to 1922 this outbreak was characterized in general by the relative mildness of the primary infection and the rarity of secondary complications. Often other signs of typical epidemic influenza, such as flush, photophobia, conjunctivitis, diffuse pharyngitis, and the characteristic leucopenia, were noted, but in the absence of acknowledged proof of the etiologic agent in clinical influenza it would be unwise to draw a close parallel between the infections of this transient outbreak and influenza of the epidemic type. A search for *Bact. pneumosintes* in these sporadic cases thus presented a new field for study and a new problem in its relation to the clinical disease.

In the 10 days that elapsed between our first contact and the failure of available material we obtained nasal and postnasal washings from 9 patients with clinical influenza and from 1 person who developed only a common cold. In several instances the patients had complained of a headache and feeling of depression for a day or two before the acute attack, so that the actual time of invasion is difficult to determine, but the washings were obtained in each instance within 24 hours of the acute onset, marked by fever and prostration in bed. We decided at the outset to follow the established routine in detail and so handled our material as follows:

The patient's nose and throat were washed out with 40 to 50 cc. of sterile, dextrose Ringer's solution. The washings were shaken with beads and divided into two portions. One sample was filtered through a new Berkefeld V candle and used to inoculate Smith-Noguchi medium and *coli* broth (10) under a vaseline seal, and was spread on rabbit blood agar plates for aerobic and anaerobic incubation. The unfiltered nasopharyngeal washings were injected intratracheally by the method of tracheotomy into stock rabbits under light ether anesthesia. The febrile and leucocytic reactions of these rabbits were carefully followed and at autopsy on the 1st or 2nd day, portions of the lung tissue of these animals were ground, and this material, filtered or unfiltered, was inoculated into Smith-Noguchi tubes and on blood agar plates. Fragments were also placed in 50 per cent glycerol, and the rabbits were examined carefully for evidence of concurrent disease.

Rabbit Passages.

Unfiltered washings from 7 of the 9 influenza patients were injected intratracheally into 13 rabbits, usually in amounts of 3 cc. 9 of these

influenza patients. The 1st generations grew so sparsely as to escape detection and the bacteria were first discovered as submicroscopic colonies in subplants on anaerobic blood agar plates. Although these 2 strains have grown well in successive generations on solid media in the anaerobic jar, they have both died out in the Smith-Noguchi tubes and repeated attempts to reestablish growth in fluid media have so far failed. The morphological similarity to *Bact. pneumosintes*, the very minute, discrete colonies on blood agar plates, and the failure to develop in successive transplants on fluid media are characteristic of the Group II organism briefly described by Olitsky and Gates in 1922 (12). These strains have not yet been grown in sufficient quantity for serological examination.

In addition to these 4 morphologically similar organisms, which may all belong to a common group, 2 other anaerobic filter passers were isolated directly from washings of influenza patients. As in the earlier studies, the identification of these 2 other organisms depended on the use of anaerobic blood agar plates on which they grow readily in visible colony form. The primary cultures were obtained in Smith-Noguchi medium; in one instance also in *coli* broth. One strain is apparently a variant of Group I, the other is similar to the organisms described as Group III (12).

Serological Reactions.

At the beginning of this investigation 6 rabbits were set aside for immunization with old strains of *Bact. pneumosintes* (C 17 and C 34) from 1919 and 1922. The organisms were grown in *coli* broth, washed, standardized, and injected subcutaneously in large doses at weekly intervals until 7 or 8 injections had been given. The rabbits then yielded serum with a complete agglutination titer of 1:160 to 1:320 against the old strains. These titers are the highest that have yet been obtained with these organisms and may indicate an increase both in agglutinogenic properties and in response to serum antibodies on prolonged saprophytic cultivation.

The 2 new strains of *Bact. pneumosintes* show a strictly specific agglutination in low dilutions, 1:2 to 1:20, of this anti-*pneumosintes* serum.

Noguchi medium, the microscopic observation of minute, Gram-negative bodies such as have been fully described (10), and the growth on anaerobic blood agar plates of microscopic, discrete, round, convex colonies with an entire edge and a colorless translucency indicated the growth of *Bact. pneumosintes* morphologically identical with the 1918 to 1922 strains.

One strain was obtained from the whole lung tissue of a rabbit injected with unfiltered nasopharyngeal washings, and consequently had not been filtered. Fortunately this rabbit was free from previous lung infection and, as in numerous cases reported by Olitsky and Gates (11), the contaminating bacteria (in this case *S. albus* and diphtheroids) in the unfiltered nasopharyngeal washings were suppressed during the rabbit passage.

The 2nd strain, also obtained through rabbit passages, was derived from the filtered lung tissue of a 2nd passage rabbit, intratracheally injected with whole lung tissue that had stood in 50 per cent glycerol for 31 days. This 1st passage lung tissue had been contaminated with a large Gram-negative bacillus which did not survive glycerolation, so that the lungs of the 2nd rabbit yielded no aerobic growth. The 2nd passage rabbit showed no fever, only a slight leucopenia (a drop in monocytes from 2790 to 2040 cells), and no gross lesions except one small surface hemorrhage in the lungs.

No primary cultures of *Bact. pneumosintes* were obtained in *coli* broth or on anaerobic blood agar plates. These media are only suitable for special purposes with well established strains. This fact emphasizes the importance of the Smith-Noguchi medium for primary cultures, and even in this medium the initial cultivation of *Bact. pneumosintes* is difficult and uncertain. A lesson may be drawn from the detection of this fastidious organism only in the 3rd generation of culture and even then only after 1 or 2 preliminary rabbit passages. It was our earlier experience that the cultivation of *Bact. pneumosintes* was more frequently successful from the lung tissues of affected rabbits than directly from the filtered nasopharyngeal washings of influenza patients.

2 other strains of anaerobic, filter-passing organisms morphologically similar to *Bact. pneumosintes* were obtained in Smith-Noguchi medium directly from the filtered nasopharyngeal washings of other

agglutinate 1 or both old strains of *Bact. pneumosintes*, and when tested, most of them (12 of 15) agglutinated 1 or both of the 1926 strains also. Considered together with the agglutination of the new strains by specific anti-*pneumosintes* rabbit serum, this evidence points to an immunological relationship as well as a morphological identity between the 1919 to 1922 and the 1926 strains. Heretofore agglutination of *Bact. pneumosintes* even after a long saprophytic existence in the laboratory has not been found in the serum of supposedly normal persons. In these tests unquestioned agglutination of old and new strains sometimes occurred. Several explanations of this phenomenon are possible, but we shall not attempt to develop any of them at this time, on the basis of the evidence available at present.

SUMMARY.

The presence of *Bacterium pneumosintes* has been demonstrated in nasopharyngeal washings from 2 patients in a sporadic outbreak of clinical influenza in New York City in March, 1926. 2 strains of bacteria morphologically similar to *Bact. pneumosintes*, but differing in certain cultural characters, and 2 other anaerobic filter-passing organisms were also isolated from the 9 patients examined.

The blood serum of 16 among 17 persons convalescent from clinical influenza, and of 6 among 10 supposedly normal persons, agglutinated 1 or more strains of *Bact. pneumosintes*.

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At intervals of 15 to 30 days after the acute onset, serum samples were obtained from 8 of the 9 influenza patients from whom washings had been taken, and from 9 other convalescents from clinical influenza. As controls 10 samples were taken from normal persons who said that within a year they had not had any acute respiratory infec-

TABLE I.
Agglutination Tests with Convalescent and Normal Human Sera.

Convalescent sera					Normal sera				
Serum dilu- tions	<i>Bact. pneumosintes</i> strain					<i>Bact. pneumosintes</i> strain			
	17	34	49	50		17	34	49	50
	1:10	1:10	1:2	1:2		1:10	1:10	1:2	1:2
1	+++	++	++	++	1	+++	++	—	—
2 ^a	—	—	—	—	2	+	—	—	—
3 ^b	++	+	++	—	3	+++	—	++	++
4	++	+++	+	+	4	+++	+	++	+
5 ^c	+++	++	++	—	5	—	—	—	—
6 ^d	++	++	++	+	6	+++	++	—	—
7	++	++	—	—	7	—	—	—	—
8 ^d	+++	++	+	+	8	—	—	—	—
9	+++	++	+	—	9	+++	—	—	—
10	+++	+	++	++	10	—	—	—	—
11	+++	+	—	+	Normal rabbit	—	—	—	—
12	+++	++	—	—					
13	+++	+	+	++					
14	+++	++	—	—					
15	++	++	++	—					
16	++	+	+	—					
17	++	—	—	—					

^a Group III organism recovered.

^b *Bact. pneumosintes* recovered.

^c Group I organism recovered.

^d Group II organism recovered.

tion diagnosed as influenza. These sera were tested for specific agglutinins, by the method previously described (13), against 2 old strains (Nos. 17 and 34), and against the 2 new strains of *Bact. pneumosintes* (Nos. 49 and 50), so far as the very limited amounts of available material permitted.

A summary of these agglutination tests (Table I) shows that the serum of only 1 patient with a clinical diagnosis of influenza failed to

animals the blood chlorides showed a decrease. The carbon dioxide-combining power did not show any constant change (Table I).

TABLE I.
Simple Jejunostomy 12 Inches below Ligament of Treitz.

Dog No.	Day after operation	Blood				Treatment
		Amount per 100 cc.			CO ₂ -combining power	
		Total non-protein nitrogen	Urea nitrogen	Chlorides		
		mg.	mg.	mg.	vol. per cent	
1	0	34.6	9.8	510	41.9	Operation
	1	37.5	10.5	460	46.6	
	2	31.2	13.0	450	40.9	
	3	49.2	18.2	460	34.3	
	4	134.0	72.7	450	33.4	Died
2	0	27.3	8.4	470	36.2	Operation
	1	30.0	14.0	490	28.5	Died
	2	52.8	26.6	470	24.7	
3	0	24.6	10.5	490	36.2	Operation
	1	40.0	20.3	430	38.0	Died
	2	129.0	66.5	370	29.4	
	3	218.0	115.5	320	29.4	
4	0	26.5	8.4	490	24.7	Operation
	1	31.6	8.4	440	29.6	
	2	67.0	22.6	360	22.8	
	3	68.3	27.9	340	29.4	
	4	92.5	46.2	340	30.5	
	5	159.0	81.2	340	25.6	Died
5	0	31.6	10.5	520	20.9	Operation
	1	37.5	9.1	430	28.5	Died
	2	115.0	43.4	430	16.2	
	3	228.0	101.5	430	21.9	
6	0	30.6	14.7	470	40.0	Operation
	1	45.0	22.4	410	40.0	Died
	2	79.8	41.3	390	43.8	

In two control dogs jejunostomy was done as indicated above and the drainage tube completely closed to prevent drainage at any time.

EXPERIMENTAL HIGH JEJUNOSTOMY IN THE DOG, WITH BLOOD CHEMICAL STUDIES.

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In a recent publication (1), we have discussed the effect of jejunostomy upon experimental obstruction of the jejunum. This work was stimulated by the interest manifested in jejunostomy in recent years as a treatment of clinical intestinal obstruction and peritonitis. The following experiments have been made to study the effect of simple high jejunostomy upon the life of the dog and the chemistry of its blood. A comparison is made between the results of high jejunostomy and low ileostomy.

Method.

All operations were done under ether anesthesia. The jejunostomy was made about 12 inches below the ligament of Treitz. A modified Witzel technique was used in some cases and in others the gut was sutured to the peritoneum and skin and opened. Low ileostomies were done with the same technique. Drainage was prompt and continuous in all cases. Blood for analysis was withdrawn from the jugular vein. All animals showing complications at autopsy were rejected. The non-protein nitrogen was determined by the method of Folin and Wu (2), the urea nitrogen by the Van Slyke and Cullen (3) modification of the Marshall method, and the carbon dioxide-combining power by the method of Van Slyke and Cullen (4). The chlorides were determined on the tungstic acid filtrate in the manner suggested by Gettler (5).

EXPERIMENTAL OBSERVATIONS.

The effects of simple jejunostomy were first studied. Six animals lived from 2 to 5 days following the jejunal drainage. In every instance the blood showed a rapid rise in the non-protein and urea nitrogen. These changes are similar to those observed in simple obstruction of the jejunum (1) but develop more rapidly. In four

TABLE III.
Ileostomy 12 Inches above Cecum.

Dog No.	Day after operation	Blood				Treatment	
		Amount per 100 cc.			CO ₂ -combining power		
		Total non-protein nitrogen	Urea nitrogen	Chlorides			
		mg.	mg.	mg.	vol. per cent		
9	0	23.0	6.3	430	39.0	Operation. Rubber tube drainage	
	1	34.5	6.3	450	42.8		
	2	33.3	9.1	460	39.0		
	3	31.6	11.2	460	40.0		
	4	24.4	11.2	460	33.4		
	5	26.8	11.9	440	35.3		
	6	23.6	7.7	480	35.3		
	7	30.9	16.8	480	30.5		
	8	37.0	11.9	530	37.2		
	9	34.5	17.5	450	32.4		
	10	41.6	11.9	520	33.4		
	11	29.1	7.7	480	29.4		
	12	29.4	9.1	540	36.2		
	13	23.3	7.0	530	34.3		
	14	23.4	6.2	580	26.6		Tube removed Dog recovered
	16	27.5	7.0	570	30.5		
10	0	33.0	7.0	460	29.6	Operation. Rubber tube drainage	
	1	50.0	11.9	450	29.4		
	2	33.3	7.0	480	29.4		
	3	31.9	17.5	440	29.6		
	4	33.0	11.2	480	39.0		
	5	33.0	16.8	460	32.4	Dog pulled tube out. Ileum sutured to skin and opened	
	6	27.3	10.5	460	36.2		
	7	27.0	8.4	440	38.1		
	8	30.9	11.2	480	31.5		
	9	31.6	8.4	480	37.2		
	10	23.0	5.6	470	32.4	Began feeding bread	
	11	31.6	2.1	450	34.3		
	12	29.1	11.2	530	34.3		
	13	33.3	11.3	490	36.2		
	14	33.3	9.1	480	30.5		
	15	22.9	6.1	500	27.5	No further blood studies. Dog died in 55 days	
	20	31.2	11.2	550	27.5		
	25	33.3	19.6	560	32.4		
	28	25.2	11.2	600	36.2		
	34	31.9	13.3	560	35.3		

TABLE II.

Jejunostomy—Tube Obstructed and No Drainage Permitted.

Dog No.	Day after operation	Blood				Treatment	
		Amount per 100 cc.			CO ₂ -combining power		
		Total non-protein nitrogen	Urea nitrogen	Chlorides			
		mg.	mg.	mg.	vol. per cent		
7	0	30.0	8.4	410	36.2	Operation	
	1	33.0	11.2	440	36.2		
	2	30.6	9.8	450	31.5		
	3	34.5	8.4	460	36.2		
	4	29.7	9.8	460	29.4		
	5	70.5	27.3	420	27.5		
	6	32.3	23.8	450	29.4		
	7	37.0	10.5	470	32.4		
	8	24.6	11.8	470	35.3		
	9		7.0	470	29.6	Feeding started	
	10	26.8	11.2	490	34.3		
	12	24.8	9.1	530	32.5		
	15	30.9	14.0	530	33.4		
	17	36.6	11.9	510	28.5		
	19	28.2	21.6	520	25.6		
	23	25.7	5.6	520	32.4		Drainage tube removed Dog well
	26	33.0	19.6	510	34.3		
8	0	25.2	11.8	490	34.3	Operation	
	1	31.6	11.8	460	31.5		
	2	30.0	7.0	370	24.7		
	3	30.0	12.6	460	30.5		
	4	23.6	7.7	470	36.2		
	5	27.3	13.3	490	27.5		
	6	27.5	20.3	480	31.5		
	7	30.3	5.6	500	31.5		
	8	21.6	7.0	520	32.4	Tube removed Feeding started Dog well	
	9	30.0	9.1	520	29.4		
	10	29.7	11.2	530	32.4		
	11	24.6	10.5	540	31.5		
	12	22.4	9.1	560	31.5		
	13	22.9	8.4	570	31.5		
	15	30.0	6.3	540	34.3		
	17	28.5	8.4	600	30.5		
	19	23.0	6.3	550	29.4	Dog well	
	23		7.0	560	33.4		

TABLE IV—*Concluded.*

Dog No.	Day after operation	Blood				Treatment
		Amount per 100 cc.			CO ₂ -combining power	
		Total non-protein nitrogen	Urea nitrogen	Chlorides		
mg.	mg.	mg.	vol. per cent			
14	7	39.5	11.2	550	28.7	500 cc. of 0.8% NaCl subcutaneously
	8	28.5	16.1	500	32.4	
	9	36.1	14.0	560	28.7	
	10	68.0	30.8	580	28.7	
	11	42.8	22.4	530	34.3	400 " " " " " "
	12	43.6	23.8	640	32.4	" " " " " "
	13	71.3	26.6	700	30.5	Died

These animals lived indefinitely without the development of the chemical changes in the blood noted in simple jejunostomy. In 10 days to 2 weeks the tubes loosened and were removed or dropped out and the wounds healed. These findings indicate that the operative procedure alone would not cause death or marked changes in the chemistry of the blood (Table II).

Ileostomy 12 inches above the cecum was done in four dogs. Three of these lived 16, 29 and 55 days and the fourth was still alive after 72 days when studies were discontinued. No constant changes in the blood chemistry were noted (Table III).

Four dogs with simple high jejunostomy were treated with sodium chloride solution. They lived from 7 to 13 days. Two were treated by giving physiologic sodium chloride solution in daily doses of 40 cc. per kilo of body weight. An effort was made in the other two to replace with sodium chloride solution the weight lost from day to day. In every instance the sodium chloride of the blood was above normal at the time of death. A definite rise in non-protein and urea nitrogen of the blood also occurred before death (Table IV).

Water by mouth was not given in these treated dogs. In spite of the quantity of liquid given subcutaneously a marked and rapid loss of weight occurred in each animal.

TABLE IV.

Simple Jejunostomy. Treatment with Sodium Chloride Solution.

Dog No.	Day after operation	Blood				Treatment
		Amount per 100 cc.			CO ₂ -combining power	
		Total non-protein nitrogen	Urea nitrogen	Chlorides		
		mg.	mg.	mg.	vol. per cent	
11	0	26.3	5.6	490	34.3	Operation
	1	42.8	10.2	430	34.3	40 cc. per kg. of 0.8 % NaCl subcutaneously
	2	42.8	9.1	460	37.2	" " " " " " " "
	3	31.6	9.1	450	29.4	" " " " " " " "
	4	33.3	9.1	500	31.5	" " " " " " " "
	5	33.3	9.1	560	25.6	" " " " " " " "
	6	29.7	6.4	580	29.6	" " " " " " " "
	7	56.4	24.5	630	30.5	Died
12	0	31.6	7.0	440	27.5	Operation
	1	26.3	8.4	450	27.5	40 cc. per kg. of 0.8% NaCl subcutaneously
	2	30.6	9.8	500	25.6	" " " " " " " "
	3		21.7	540	23.8	" " " " " " " "
	4	25.0	13.3	560	25.6	" " " " " " " "
	5	25.0	11.9	470	25.6	" " " " " " " "
	6	36.1	14.7	600	22.8	" " " " " " " "
	7	34.1	18.9	690	20.9	Died
13	0	28.2		510	40.0	Operation
	1	29.1	10.5	400	36.2	950 cc. of 0.8% NaCl subcutaneously
	2	32.3	5.6	550	34.3	
	3	25.0	9.8	490	31.5	1920 " " " " " "
	4	32.3	14.8	560	10.7	
	5	30.9	19.6	600	20.0	
	6	60.0	11.2	700	21.9	700 " " " " " "
	7	114.0	49.8	750	16.2	Died
14	0	34.9	9.1	430	31.5	Operation
	1	33.3	10.5	430	32.4	800 cc. of 0.8% NaCl subcutaneously
	2	33.0	5.6	510	30.5	
	3	31.2	7.9	490	29.4	600 " " " " " "
	4	26.1	11.9	530	30.5	
	5	28.0	9.1	520	25.6	
	6	41.6	22.9	640	18.1	500 " " " " " "

4. Van Slyke, D. D., and Cullen, G. E., *J. Biol. Chem.*, 1917, xxx, 289.
5. Gettler, A. O., *J. Am. Med. Assn.*, 1921, lxxvii, 1652.
6. Haden, R. L., and Orr, T. G., *J. Exp. Med.*, 1923, xxxvii, 365.
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8. Haden, R. L., and Orr, T. G., *J. Exp. Med.*, 1923, xxxviii, 55.

DISCUSSION.

Dogs with simple high drainage of the jejunum lived from 2 to 5 days. This is a shorter average length of life than with simple obstruction at the same location. The chemical changes of the blood are similar to those occurring in high intestinal obstruction (6). They differ however from pyloric (7) and duodenal obstruction in that the carbon dioxide-combining power does not show any constant change and the chlorides do not show such a marked fall.

Animals with simple jejunostomy and closure of tube to prevent drainage proved that the operation done was not the cause of death in these cases.

Ileostomy 12 inches above the cecum did not produce the profound disturbance of high jejunostomy. It is probable that some dogs would live indefinitely with low drainage of the ileum.

Treatment with sodium chloride solution definitely prolongs the life of dogs with high jejunostomy. The cause of the rapid death in high jejunostomy apparently differs from that due to high intestinal obstruction. Sodium chloride solution will not protect in this condition to as great an extent as in high intestinal obstruction (8).

Whether cause of death is due to dehydration, toxemia, loss of chlorides or loss of other important elements is not known. Fasting is not to be considered because of the short duration of life.

CONCLUSIONS.

1. The effect of high jejunostomy upon the life and chemical changes of the blood of dogs is here reported.

2. Sodium chloride solution administered in sufficient quantity after high jejunostomy prolongs the life of dogs.

3. Such experimental findings as these warrant a careful clinical study of the effects of high jejunostomy so frequently used in the treatment of acute intestinal obstruction.

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bacteria, and stain much less intensely, it is not surprising that so little is known of their exact relation to the tissues of man and experimentally infected animals.

It is believed that heartwater affords an unusual opportunity for securing information on this particular aspect of the general problem of *Rickettsiæ* and of their relation to disease.² As already noted degenerative vascular lesions are almost entirely absent in heartwater, and the *Rickettsiæ* themselves, while very sparsely distributed in the tissues as compared with some bacteria in other conditions, are nevertheless much more abundant than the *Rickettsiæ* in any of the above mentioned infections. Another advantage offered by the heartwater *Rickettsiæ* is that they may be colored, without great difficulty, by a variety of stains in addition to that of Giemsa on which chief reliance must be placed for the study of the other *Rickettsiæ*.

Despite the unusual facility with which the heartwater *Rickettsiæ* may be observed and studied, they resemble other *Rickettsiæ* closely in many important particulars. Like them they are very small, Gram-negative organisms (resisting attempts at cultivation on artificial media) which are found principally in the endothelial cells of the blood vessels of diseased animals. All are transmitted by the bites of insects or arachnids, and the conditions to which they give rise are similar in so far as they are acutely febrile, the nervous system is frequently involved and they confer an immunity lasting for several years or for life. The chief point in which heartwater differs from typhus fever and Rocky Mountain spotted fever is in the absence of cutaneous lesions, which is probably correlated with the circumstance already mentioned, that there is but slight vascular involvement.

EXPERIMENTS.

Most of the animals studied were infected by the intrajugular inoculation of blood from cases of heartwater; but some were infected by allowing ticks, carrying the virus, to feed upon them. They were sacrificed at appropriate intervals during the incubation period, and febrile stages, and during convalescence (in the rare cases which did not terminate fatally). A detailed account of the experiments, together with the autopsy reports, has already been given in the first two papers.

Rickettsiæ in the Circulating Blood Stream.

That the virus, and presumably therefore the *Rickettsiæ*, are present in the blood stream during the febrile stages and for about 6 days after the temperature has commenced to decline in animals which have not

² Cowdry, F. V., *Arch. Path. and Lab. Med.*, 1926, ii, 59.

STUDIES ON THE ETIOLOGY OF HEARTWATER.

III. THE MULTIPLICATION OF *RICKETTSIA RUMINANTII* WITHIN THE ENDOTHELIAL CELLS OF INFECTED ANIMALS AND THEIR DISCHARGE INTO THE CIRCULATION.*

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PLATE 31.

(Received for publication, August 25, 1926.)

The first two of these studies¹ dealt with the discovery of a new species of *Rickettsia* in the tissues of sheep, goats and cattle suffering from heartwater and in the ticks which transmit it. In this third paper, it is proposed to describe in detail the behavior of the *Rickettsiæ* after they have entered the endothelial cells of the blood vessels of infected animals.

The chief obstacle to the acceptance of *Rickettsiæ* as the inciting agents of disease in man is the difficulty of demonstrating their existence clearly and regularly in the lesions. This is almost insurmountable in the case of trench fever, because it is seldom if ever, fatal, so that material for examination cannot be collected post mortem, unless death should accidentally occur at a propitious moment from some other cause, which in practice has not happened. In two other arthropod-transmitted diseases—typhus and Rocky Mountain spotted fever—*Rickettsiæ* are undoubtedly present in the tissues, but only in very small numbers. Their distinctive properties are obscured by extensive vascular degenerations resulting in the formation of many granules which, like them, are colored feebly by basic dyes. Since, moreover, all three species of *Rickettsiæ* are smaller than most

* Sixth contribution by the South African Expedition of The Rockefeller Institute for Medical Research.

The experiments were made and material collected at the Government Laboratories at Onderstepoort and cordial thanks are due to the Government of the Union of South Africa, to Sir Arnold Theiler and to the members of his staff for the many courtesies extended. The study herein reported was made after my return to the United States.

¹ Cowdry, E. V., *J. Exp. Med.*, 1925, xlii, 231, 253.

Before considering the probability of sequence in penetration, reference must be made to an observation which was briefly mentioned in the first paper, namely, that many spherical aggregates of *Rickettsiæ* may develop within a single endothelial cell. Their number may reach as many as eight. In Fig. 1 six are illustrated within the limits of a section only 3 microns in thickness. The colonies invariably remain distinct and never fuse, even when closely applied to each other.

The clumps inhabiting one cell are seldom of the same size. Their dimensions may vary from about $0.5\ \mu$ to $25.0\ \mu$ as observed after fixation and staining, and the number of *Rickettsiæ* from two to several hundreds. Sometimes the *Rickettsiæ* in the smaller ones are slightly larger and color more deeply than those grouped in the larger masses (Figs. 1 and 6). Although such a distinction is by no means of constant occurrence, it does seem probable that the smaller colonies are younger than the larger ones,—that they had less time to multiply—in other words, that there has, in fact, been a sequence in the entry of *Rickettsiæ*.

Just why the *Rickettsiæ* should enter some endothelial cells in marked preference to others is not known. It does not seem to take place *pari passu* with the phagocytosis of broken down erythrocytes, for example. In heartwater there is very little taking up of erythrocytic debris by the endothelial cells of the cerebral cortex, and kidney, which nevertheless harbor *Rickettsiæ* more constantly than the cells of any other tissues. In the rare cases when it is phagocytized in these localities the cells which do so have never been observed to accumulate *Rickettsiæ* as well, but it would be unsafe to assert that such a double acquisition is impossible. It is conceivable that there may be an element of antagonism in the two processes. Thus, the *Rickettsiæ* were never seen in the endothelial cells of the liver, and they are of very inconstant occurrence in those of the spleen, both of which are noted for the avidity with which they phagocytize erythrocytes and materials of foreign origin generally. Neither is the liver the best place to search for the *Rickettsiæ* of typhus and Rocky Mountain spotted fever, although they do occur in it sparingly. Experimental blocking of the endothelium in heartwater with particulate matter might perhaps modify its reaction to the *Rickettsiæ*.

succumbed, was shown by the inoculation of blood into other susceptible animals. But although many attempts were made, it was not possible to discover the *Rickettsiæ* in blood smears. This failure corresponds with the experience of investigators while studying the blood of cases of trench fever and of typhus fever. A few heartwater *Rickettsiæ* were, however, detected within the vascular lumina of tissues examined in sections. The most probable explanation is that they do not occur in the blood stream in large masses but as single individuals, or perhaps in the form of very small clumps (Fig. 7). Obviously even the most careful differentiation of the stain might completely bleach single elements of such small dimensions (only about $0.25\ \mu$ in diameter), whereas the large aggregates within the endothelial cells would probably retain the dye more tenaciously, owing to its reduced rate of diffusion.

Penetration of the Rickettsiæ into the Endothelial Cells.

The actual passage of *Rickettsiæ* through the cell membrane was never seen, but there is reason to believe that several may enter a single cell, most likely in sequence.

The sites of entry and the foci of subsequent multiplication are variable. They may penetrate the cytoplasm at a point where it consists of a thin film almost invisible microscopically, relatively remote from the nucleus. Or, they may gain admittance to the cytoplasm lying between the endothelial nucleus and the lumen of the blood vessel (Fig. 2). In rare cases they may migrate through the cytoplasm to a point on the opposite side of the nucleus, that is to say between it and the periphery, remote from the lumen (Fig. 5).

Multiplication in such a location, proximal to the endothelial nuclei, has not thus far been reported in the case of any of the other pathogenic *Rickettsiæ*. Yet it might be expected to occur, because the *Rickettsiæ* of both typhus and Rocky Mountain spotted fever possess much greater power of penetration than the heartwater *Rickettsiæ*. They even enter the successive vascular tunics and frequently gain an extravascular position, whereas those of heartwater are apparently definitely and invariably confined to the vascular lumen and its lining endothelium.

surface indicates that their growth pressure is about equal. If it were greater in one than in the other, only one would become flattened (*i.e.*, that in which the pressure was least), or else the one endowed with the largest expanding force would indent the other. It is possible, thus, to ascertain that the growth pressure of small colonies is approximately the same as that of larger ones.

Throughout this period of multiplication the morphology and staining reactions of the *Rickettsiæ* vary only within the limits already alluded to. Figs. 3, 4 and 6 illustrate their appearance by Giemsa's method; Figs. 2 and 5 by hematoxylin and eosin; Fig. 7 by Loeffler's methylene blue; and Fig. 1 after staining with eosin and methylene blue. The variability in color of some of the *Rickettsiæ* is probably caused by fortuitous differences in the rate of extraction of the stains, some of the *Rickettsiæ* being nearer than the others to dye-holding materials or to the surface of the section.

Thus, in their tinctorial properties also, they resemble the *Rickettsiæ* of typhus and Rocky Mountain spotted fever, though they are more easily stained. In all three the differentiation into red- and blue-staining materials, which may be observed in smears of the arthropod vectors, is not distinguishable in sections of infected mammalian tissues.

That the *Rickettsiæ* of heartwater are organisms possessed of definite internal organization, is shown by the fact that they retain their spherical shape and do not tend to flatten out when they come in touch with the cell wall and other cellular components. Yet with a very high magnification (of 4000 diameters or more) their outlines become more hazy than do those of the mitochondria, for instance. This would lead one to suppose that, if they possess an organized limiting membrane, it is of somewhat rudimentary character as compared with that of typical bacteria.

Only in an animal's tissues which have not been promptly excised after death, are any signs of the degeneration of *Rickettsiæ* to be noted. When, under such conditions, degeneration sets in the *Rickettsiæ* begin to exhibit considerable variability in size and to lose their peculiar tinctorial properties. The forces which have previously kept them spacially arranged in respect to their fellows cease to operate and they tend to agglutinate in rather amorphous masses. Though

The *Rickettsiæ* of heartwater resemble those of typhus fever in this predilection which they have for the brain, while they differ in other minor points of distribution, such as their failure to accumulate in very large numbers in the skin, or the testicle, and the peculiar attraction which the kidney seems to exercise over them.

Growth of the Rickettsiæ within the Endothelial Cells.

A single isolated *Rickettsia* has never been seen within an endothelial cell; but in Fig. 5 a *Rickettsia* in the act of division, or two *Rickettsiæ* in especially close contact, are illustrated. In very thin sections ($1\ \mu$) it is possible to distinguish similar formations suggestive of simple transverse fission, and it is probable that this process takes place commonly and at a very rapid rate. Sometimes the *Rickettsiæ* are congregated at the periphery of the colony in such a manner as to suggest the interpretation that they divide in a plane perpendicular to its surface and that the growth is peripheral rather than central (Fig. 3).

Some striking examples of growth pressure were found. Fig. 2 illustrates a colony which has developed between the nucleus and the vascular lumen. While growing it has pressed upon the nucleus, forming a marked concavity in it. A rarer instance, which, indeed, has only once been observed so clearly, is still more significant (Fig. 4). It consists of two colonies of approximately the same size (and age) which have developed at opposite poles of the nucleus of an endothelial cell. When the *Rickettsiæ* first commenced to multiply, this nucleus was presumably flattened by arterial blood pressure in a direction parallel to the length of the vessel, like its neighbors. The pressure which the *Rickettsiæ* have exerted has completely altered its position so that it has been forced to stand on its head, so to speak. This can only mean that the growth pressure of the *Rickettsiæ* actually exceeds the arterial blood pressure because it is sufficient to cause a nucleus, which is ordinarily flattened by the blood pressure, to expand directly against it. To the best of my knowledge no alteration like this is produced through the phagocytosis of lifeless materials.

The observation that when two colonies grow toward each other and come in contact the area of separation between them is a plane

the interface between the fluid contents of the spaces and the cytoplasmic environment. It is interesting to note that neighboring spaces communicate with one another, as illustrated in Figs. 1 and 6; also, that a single space may house more than one colony of *Rickettsia* (Fig. 1).

The reason why these colonies of *Rickettsia*, suspended in a fluid medium within a single vacuole-like container, or communicating system of containers, of microscopic dimensions, should retain their individuality instead of coalescing to form a larger mass, is not easily discovered. One would expect them to be thoroughly mixed by the continual changes in size of the pulsating vascular walls, but they are equally discrete in veins, which do not pulsate, as in arteries.

In sectioned mammalian material, it has not been possible thus far to bring to light any indications of the existence of a matrix, or binding material, which might restrict or retard the movement of the individual organisms forming a colony. But, in the second paper, on the *Rickettsia* of heartwater as they appear in infected ticks,³ some clumps of *Rickettsia* were found to be embedded in such a ground substance which differed slightly in staining properties from the cytoplasm of the host cells. This was best seen after formalin fixation. When, moreover, portions of the tick's alimentary tract were teased out in an approximately isotonic medium the individual *Rickettsia* in a clump were found to cohere together despite considerable mechanical traction. Two possibilities therefore present themselves; either a matrix of this kind is absent in the tissues of infected mammals, or else it is present in such a form that it eludes our methods of demonstration. It may be an inconstant accompaniment of intracellular *Rickettsial* growth, and exist in very small amounts in the case of *Rickettsia prowazeki* in which the "globular massing" is so much less distinct.

Discharge of Rickettsia from the Endothelial Cells into the Blood Stream.

After the incubation period has passed and the febrile reaction is at its height, the endothelial cells often rupture and discharge their contents into the circulation (Figs. 4 and 6). Sometimes the broken

³ Cowdry, E. V., *J. Exp. Med.*, 1925, xlii, 253.

unquestionably dead, they may still be recognized for 6 hours or more after the death of the animal containing them, chiefly by their topographic relationship to the blood vessels.

Reaction of the Endothelial Cells to the Presence of the Rickettsiæ.

Unless the living endothelial cells contain *Rickettsiæ*, they show no signs of injury. In this respect the endothelial reaction in heartwater differs sharply from that which may be observed in typhus and Rocky Mountain spotted fever. In these two diseases the endothelial cells in which the *Rickettsiæ* are absent become altered almost as regularly and to about the same degree as those which harbor many of them.

When the *Rickettsiæ* of heartwater do enter the endothelial cells and multiply within them, they do not cause very serious injury. The principal alteration which the cells undergo is great mechanical distention to accommodate the *Rickettsiæ* in large numbers. Their nuclei seldom, if ever, show definite signs of injury. Karyokinetic figures are of very rare occurrence and there is no evidence of amitosis. It is possible that the mitochondria and Golgi apparatus in the cytoplasm are altered; but they cannot be greatly damaged, because the cells continue to live. No signs whatever of basophilic degeneration, like that which occurs in typhus and Rocky Mountain spotted fever, were seen. Indeed, the cytoplasm, although swollen and greatly increased in extent, reacts in the usual way to all ordinary stains except for certain foci, in which the *Rickettsiæ* are multiplying, which become chromophobic.

These vacuole-like spaces containing *Rickettsiæ* are filled with a clear watery fluid, probably saline in nature, as the action of a wide range of fixatives fails to reveal any coagulable substance in it. The margins of these spaces are sharply defined and do not grade into the surrounding cytoplasm. Morphologically the spaces do not differ from areas of intracellular digestion containing broken down red blood cells, but biochemically they are probably dissimilar, for the reason that there can be but little in common between the process of intracellular digestion and the conditions which facilitate the multiplication of *Rickettsia*-like organisms.

The *Rickettsiæ* of heartwater are usually observed clumped in the central parts of these spaces. They do not become applied to

of artificial media suitable for the cultivation *in vitro* of the heartwater *Rickettsiæ* and conceivably for those of typhus and Rocky Mountain spotted fever also. Many difficulties would be encountered in making such an analysis, but indicators might be of service, especially the *in vivo* synthesis of Prussian blue. The oxydase reaction should be applied. The fact that multiplication appears to attain a maximum during the height of the febrile period, when the temperature often reaches 107° or 108°F. may be significant taken in conjunction with the fact that multiplication is also rapid in the body of the tick at a temperature about 30° lower. Additional studies on the matrix which seems to bind together the individual *Rickettsiæ*, which compose a colony, might also bring to light information regarding a medium which would justify further work along the lines of artificial cultivation.

Another feature which all pathogenic *Rickettsiæ* possess in common is the habit of becoming parasitic in the vascular endothelia. This is the usual location in human and mammalian tissues of the *Rickettsiæ* of Rocky Mountain spotted fever, in which condition, however, they may spread to the media and adventitia and occasionally to parenchymatous elements such as liver cells.⁵ The *Rickettsiæ* of typhus fever are somewhat more restricted, being found only in the mononuclear cells of the perivascular nodules in addition to the endothelium; while the *Rickettsiæ* of heartwater are the most specific of all, since their location is confined, as far as can be ascertained, without exception, to the endothelium.

This restriction of a pathogenic microorganism to a single type of cell has recently been claimed for the tubercle bacillus by Sabin and her coworkers.⁶ She believes that the tubercle bacillus attacks a specific kind of cell—the monocyte—penetrates into its interior and multiplies within it. The evidence for intracellular multiplication is conclusive. Maximow⁷ has observed the process in the living cells of tissue cultures; but he has found that other cells, as well as the monocyte, are invaded, and some believe that the monocytes do not differ fundamentally from

⁵ Nicholson, F. M., *J. Exp. Med.*, 1923, xxxvii, 221.

⁶ Cunningham, R. S., Sabin, F. R., Sugiyama, S., and Kindwall, J. A., *Bull. Johns Hopkins Hosp.*, 1925, xxxvii, 231.

⁷ Maximow, A. A., *J. Infect. Dis.*, 1924, xxxiv, 549.

edges of the cell membrane may be distinguished so that it is certainly not a case of the entry of *Rickettsiæ*. That we are not dealing with a dragging out of the *Rickettsiæ* by the microtome knife (as occasionally happens in the case of resistant bodies, like nucleoli) is indicated by the absence of scratches and by the observation that in one and the same section the discharge may take place in several directions. As far as our microscopic evidence goes, these liberated *Rickettsiæ* have every appearance of being just as viable as those which originally entered the cell. No parallel instance of the discharge of *Rickettsiæ* into the blood stream has been reported in Rocky Mountain spotted fever or in typhus fever. A special search which I have made myself of tissues very kindly given to me by Drs. Wolbach, Rajchmann and Nicholson has failed to reveal any indications of a similar process.

Even with localized cellular injury of this extent and degree, the nucleus remains apparently normal and the cell continues to live. This normality of the endothelium constitutes another point in favor of the conclusion that the *Rickettsiæ* live and actively multiply within the vascular endothelial cells. By contrast, the extensive endothelial injury in typhus and Rocky Mountain spotted fever seems to be correlated, at least in part, with the death of many *Rickettsiæ* within the cells and the resultant liberation of substances which may be poisonous. In other words, the endothelial inclusion of *Rickettsiæ* is not so much a mechanism of defense in heartwater as it appears to be in these other diseases.

DISCUSSION.

The *Rickettsiæ* of heartwater exhibit certain features typical of other pathogenic *Rickettsiæ* with almost exaggerated clearness.

The most characteristic appearance of *Rickettsiæ* in human lesions is, according to Wolbach, Todd and Palfrey,⁴ their tendency to globular massing. In heartwater this property of forming spherical clumps within the endothelial cells is even more conspicuous. These spherical masses, or colonies, grow within vacuole-like cytoplasmic spaces filled with a clear fluid. Further data are needed concerning these spaces, because clues might thereby be secured for the preparation

⁴ Wolbach, S. B., Todd, J. L., and Palfrey, F. W., The etiology and pathology of typhus, Cambridge, 1922.

FIG. 1. Cerebral cortex fixed in Regaud's fluid and stained with eosin and methylene blue. Several colonies of *Rickettsiæ* are seen in the cytoplasm to the left of the nucleus. They are of variable size. In the smallest, a single organism is represented which is slightly larger than the others, is colored rather more intensely and seems to be in the act of division.

FIG. 2. Cerebral cortex fixed in Regaud's fluid and stained with hematoxylin and eosin. The *Rickettsiæ* are feebly colored. They are contained in an unusually large vacuole-like space (distal to the nucleus, *i.e.*, between it and the lumen) the upper margin of which can be clearly distinguished. Through growth pressure they have indented the nucleus.

FIG. 3. Cerebral cortex fixed in Regaud's fluid and stained by Giemsa's method. A colony of *Rickettsiæ* is represented at one pole of the nucleus. Organisms which seem to be dividing are oriented with plane of division at right angles to the margin of the vacuole.

FIG. 4. Pancreas fixed in Regaud's fluid and stained by Giemsa's method. There are two colonies of *Rickettsiæ*, one on either side of the nucleus. Their growth has exerted sufficient pressure to cause the nucleus to alter completely its position by projecting directly toward the lumen in a direction parallel to pressure exerted by the blood stream. A few of the *Rickettsiæ* are being discharged into the lumen.

FIG. 5. Cerebral cortex fixed in Regaud's fluid and stained by hematoxylin and eosin. Here the *Rickettsiæ* are multiplying in a very unusual position, proximal to the nucleus and remote from the lumen.

FIG. 6. Cerebral cortex fixed in Regaud's fluid and stained by Giemsa's method. Two colonies of *Rickettsiæ* are illustrated. Those comprising the smaller one are stained more intensely than the others and are noticeably larger. Some *Rickettsiæ* are escaping from the larger colony into the blood stream.

FIG. 7. Cerebral cortex fixed in Regaud's fluid and stained with Loeffler's methylene blue. Two *Rickettsiæ* embedded in some chromophobic material are seen in the lumen between the erythrocytes.

the macrophages,⁸ or clasmatoocytes, as they are often called. The case of heartwater is more concise, for there can be no dilemma in the identification of endothelium. While the *Rickettsiæ* of heartwater may apparently live and multiply within the endothelial cells, there is no evidence to indicate that their action stimulates the endothelial cells to divide and increase in number in a fashion comparable with the influence exercised by ingested foreign materials, such as the tubercle bacilli, upon the monocyte.

It is interesting to note that, although in heartwater the endothelial cells remain living and normal and are so heavily charged with *Rickettsiæ* that they may be considered to be marked by their presence, the free large mononuclear cells of the circulating blood (monocytes, endothelial leucocytes, etc.) which Mallory and his associates believe to be developed directly from the endothelium, remain wholly devoid of *Rickettsiæ*, or at least contain *Rickettsiæ* so infrequently that they have thus far escaped observation.

SUMMARY.

The *Rickettsiæ* of heartwater are more definitely restricted to the vascular endothelial cells of infected animals than are those of typhus or Rocky Mountain spotted fever. They likewise form more pronounced spherical colonies within the cells. Their presence does not injure the endothelial cells to a degree at all comparable with that caused by the other pathogenic *Rickettsiæ*. The rupture of endothelial cells and discharge of *Rickettsiæ*, which are apparently viable, into the circulation constitute phenomena not thus far reported in the case of typhus or Rocky Mountain spotted fever.

EXPLANATION OF PLATE 31.

All the figures were drawn at the level of the table with a 1.30 aperture apochromatic objective of 1.5 mm., compensating ocular 18 and camera lucida. They have been reproduced without reduction so that, as they appear, they represent a magnification of 4033 diameters. They have all been made from tissues of Goat 4510.⁹

⁸ Lewis, M. R., Willis, H. S., and Lewis, W. H., *Bull. Johns Hopkins Hosp.*, 1925, xxxvi, 175.

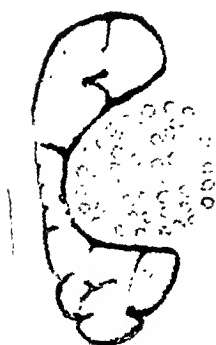
⁹ For details of temperature reaction and postmortem examination, see Cowdry, E. V., *J. Exp. Med.*, 1925, xlii, 231.



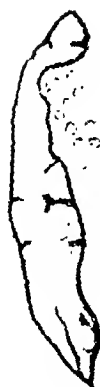
1



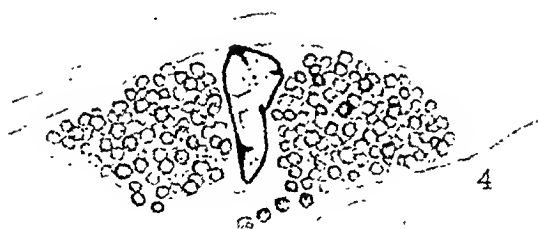
2



3



5



4



7

Exc. del.

6

thickness of the integument and contained, in addition to epidermis and corium, a varying quantity of fat and the thin layer of striped muscle which in the mouse and rat extends superficially over almost the whole body. It was frequently impossible to remove such large pieces without cutting cutaneous vessels that supplied neighboring regions, and in consequence of this occurrence the grafts often failed to become vascularized promptly and perished after some days. In females the mammary gland proved a serious complicating factor, and after a few trials males alone were used. The best results were had when the graft was not separated all at once from its surroundings but left attached by an isthmus that was severed only after the major portion had been sewn in place. By this procedure the swabbing of the raw surfaces with saline solution to prevent drying was reduced to a minimum; but great care had to be taken in the dissection else some of the loose web of tissue connecting the skin with the underlying parts escaped being cut, with result that total ischemia of the graft did not develop. For the sewing a fine curved needle carrying a single strand of the three which are wound into No. 2 surgeon's silk was employed; and an over and over stitch. Perfect approximation of the wound edges proved essential, for when a part of the wound bed was left exposed necrosis usually spread from it (5). Aseptic conditions were maintained during the operation; and no dressing was put on afterwards. The mouse had to be prevented from removing stitches and graft however. This was accomplished by passing its head through a hole in the center of a flat disc of pasteboard. The projecting collar thus formed, which stood out like the ruff of a clown, did not interfere with the animal's movements but kept it from gnawing at the graft. The stitches were removed on the day following the operation, and thereafter for nearly a week the attachment of the implanted skin was but frail.

Each day the mouse was injected into the peritoneal cavity with 0.5 cc. of a watery 4 per cent solution of phenol red, made as follows: 2 gm. of the phthalein (Hynson, Westcott and Dunning) is ground to a paste with a little water, 9.7 cc. of N/1 NaOH added, and then water to 50 cc. Such a solution is at pH 7.4 (as determined with the potentiometer) and approximately isotonic with 0.9 NaCl. The quantity of alkali employed somewhat exceeds that required, on theory, to bring the indicator to the hydrogen ion concentration mentioned, presumably because of acid impurities in the material. In order to effect the injection into the mouse without a struggle that might have entailed separation of the graft it was briefly anesthetized by dropping it into a jar containing cotton soaked in ether.

During most of the 1st week after implantation the graft appeared pallid,—save when vitally stained,—and bloodless. As is well known vessels begin to penetrate into transplanted skin during the second 24 hours, but there is certainly no effective circulation of blood for a much more considerable period in grafts of the size studied in the

THE RELATIVE REACTION WITHIN LIVING MAMMALIAN TISSUES.

VI. FACTORS DETERMINING THE REACTION OF SKIN GRAFTS; A STUDY BY THE INDICATOR METHOD OF CONDITIONS WITHIN AN ISCHEMIC TISSUE.

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PLATE 32.

(Received for publication, June 25, 1926.)

In previous papers from this laboratory a technic has been described for the examination of tissues vitally stained with litmus or with indicators of the phthalein series (1), and some observations on the apparent reaction within various organs have been recorded (2). Recent control tests on the influence of tissue materials upon the colors manifested by phthaleins staining them have shown that these colors are not conditioned to any important degree by "salt and protein errors," but that they are really indicative of the prevailing pH (3). By means of vital staining with selected indicators one should be able to apprehend, and may even come to understand, certain physiological states inaccessible to study by approved quantitative procedures. One such state, that of "outlying acidosis," has already been briefly reported upon (4). The present paper is concerned with the conditions in a tissue, the skin, which survives when its blood supply has been cut off. The problem has both practical and theoretical ramifications. In corollary to it the changes which result from superimposed injuries to the tissue elements will be considered.

The Vital Staining of Skin Grafts with Phenol Red.

Male white mice of 25 to 30 gm. were shaved over the sides and back, from fore legs to hind, and under ether anesthesia pieces of the skin were excised, midway between axilla and groin, and at once sewn back in place again. These pieces were roughly circular and from 1.0 to 1.6 cm. in diameter. They had the entire

orange ordinarily, after a little less than an hour in all, and then appears even (Fig. 2). The hue of many mice will already have begun to fade, owing to elimination of the phthalein into bile and urine; but long after the general decoloration has taken place, a process usually completed by the end of $2\frac{1}{2}$ hours, the graft remains brilliantly tinted (Fig. 3). It is still yellow more than 5 hours after the injection; for the dye leaves the avascular tissue far less rapidly than it enters it.

These are the happenings whenever the animal is stained during the first 3 or 4 days after the implantation,—and it can be stained again and again without evident injury to either it or the graft. Later, as the new vessels become effective and the penetration of the phenol red into and out of the implanted tissue does not lag so noticeably, the color of the graft comes day by day to have less of yellow and more of red in it. By the end of a week the “take” is usually perfect, and the phthalein coloration differs practically not at all from that of the host.

Abnormal Reaction of the Grafts.

The color of the stained graft seems to indicate that it is acid as compared with the normal skin round about; and there is every reason to believe that this is the actual case. Phenol red is a stable indicator, not liable to error through its association with tissue components (8) with the important exception of the proteins of plasma. Kendall (9) states that some destruction of it by reduction occurs within the organism; but the amount changed in this way is negligible when large quantities are given, as was the case in the work here reported. Dr. D. R. Drury, of the laboratory staff, has recovered from the urine of a rabbit more than 96 per cent of the phenol red required to stain it vitally, and a part of the missing 4 per cent was present in the feces, which were not extracted. For nearly 40 years (10) the fact has been generally recognized that acidity develops in tissues when their blood supply is interfered with. If a string is tied tightly around the shaven leg of a rat stained with phenol red the color of the leg turns from red to orange within a few minutes, only to become red again shortly after the cord is loosened and circulation resumed. If one evert a flap of living subcutaneous tissue vitally stained with the dye it turns from

present work. This is evident not merely from the aspect of the graft under ordinary conditions but from the slowness with which it colors up after an intraperitoneal injection of phenol red. On the 3rd day after operation it stains no more rapidly than on the 1st. Thereafter, though, it lags in this respect less markedly as compared with the skin round about, and by the end of a week, in successful instances, it colors as fast as the latter.

The rate of coloration provides enlightening data on the fluid interchange taking place within engrafted tissue. Phenol red is very highly diffusible, coloring mice deeply within a few minutes after an intraperitoneal injection; and the brilliant, ruddy color of the stained animals is due for the most part to an extravascular penetration of the dye. This has been shown by perfusing the stained animal until free of blood, with warm Locke's solution introduced into the beating heart, after the inferior vena cava has been snipped across (6). But the fact emerges even more strikingly from the observations on the skin grafts of the present work. These color deeply, evenly, and surprisingly fast with the phthalein at a time when it is certain no blood can reach them, that is to say within a few minutes after they have been separated and sewn in place again. The hue they manifest is referable to staining of the subepidermal tissue fluids and tissues, especially the corium. Muscle stains but slightly with the dose of indicator I have employed; fat scarcely at all; while the epidermis is so thin and so lightly stained that it may be dismissed from account.

In recording the hues of animal and graft Ridgway's "Color standards and nomenclature" (7) has proved of great service. Wherever the hues provided by this book are mentioned in describing the findings italics will be employed. For the matching, one of the standards at a time was exposed through a hole cut in a sheet of white paper, and compared with the skin color. A normal mouse of 28 gm. given 0.5 cc. of phenol red ordinarily becomes deeply stained within 15 minutes, and reaches a maximum color, one varying between *jasper red* and *eugenia red*, about 30 minutes after the injection. The healthy avascular graft usually remains entirely unstained for from 10 to 15 minutes (Fig. 1) after the injection,—a staring, pallid patch in the midst of the red body surface,—and then it begins to turn yellow here and there. The staining reaches a maximum intensity, *apricot*

to heap up, just as they accumulate in subcutaneous areas temporarily deprived of circulation through the vascular contraction following on a local injection of epinephrine (13). The cells of injured tissue on the other hand not only work less actively, or dying, cease to work at all, but to a greater or less degree they lose that semipermeability which characterizes them during life. As result they are penetrated by the alkaline lymph which acts to sweep away such acid products as may arise through autolysis, and, coming gradually into equilibrium with this fluid, they tend to approach it in reaction. One may doubt whether the interstitial fabric which forms so large a part of the corium, though staining deeply with the phthalein (14), possesses life of its own in any proper sense. One must think of its reaction as determined preponderantly by the activities of the cells dispersed through it.

Grafts Injured Experimentally Are Alkaline.

To test this explanation of the findings pieces of skin of the usual size were damaged prior to implantation. The intention was to inflict the minimum insult that would ensure an eventual failure of the graft. Heat was employed in some cases, but repeated freezing and thawing proved better for the purpose.

In order to heat the pieces of skin they were folded upon themselves with the raw surface inwards, and placed far down in test-tubes already in a water bath at 50–51°C. The grafts adhered to the sides of the tubes which had moist gauze at the bottom to prevent drying. They were heated for from 7 to 10 minutes.

When a graft was to be frozen and thawed it was spread upon a sterile mica slide with its raw surface against the latter. To prevent drying the edges were sometimes folded under; but more usually a drop of salt solution, or Ringer's solution, was run around the edge of the tissue. The slide was then placed on the freezing disc of a microtome, the tissue frozen solid by the escape of CO₂, and the preparation removed and thawed at once with the warmth of the hand or that of a metal plate at about 38°C. The processes of freezing and thawing were carried out three or four times as rapidly as possible, and the graft was replaced in position. Meantime the skin defect on the body of the animal was covered with a sponge moist with salt solution or Ringer's solution.

It proved easier to return the grafts to their original position when they were asymmetric or had been cut with a slight projection at one point in the periphery that fitted into a notch in the skin.

Heating to a temperature of 50°C. for 10 minutes should have killed the tissue,

red to purple in proportion as carbon dioxide escapes from the raw surface; and the purple can be converted to orange-yellow by brief exposure of the tissue to an atmosphere of CO_2 (11). These are simple instances illustrating the readiness with which the phthalein reacts under *in vivo* conditions and showing that it reacts characteristically.

One can appraise the color of the stained body surface of the living animal by oiling it, placing over it an Autenrieth wedge filled with water, and comparing the color as thus viewed with that obtained by superimposing a similar wedge, filled with an appropriate buffer solution colored with phthalein, over the oiled and shaved skin of a normal animal. By varying the buffer solution and moving the colored wedge until precisely the right depth of fluid is obtained, one can closely approximate the hue of the stained tissue. *Apricot orange* corresponds with a pH of about 6.8, *jasper red* with pH 7.4, and *eugenia red* with pH 7.6. The actual figures can be disregarded. It is their relation to each other which tells the story. Evidently vigorous skin grafts of the mouse are, relatively speaking, about pH 0.6 more acid than the normal skin, and they are able to survive this state of affairs for several days. The acidity is referable, at least in the beginning, to the elements proper to the tissue, not to the many cells that wander in (12), as is sufficiently shown by its development within an hour after implantation of the graft.

Vigorous Grafts Are Acid, Weak Ones Alkaline.

At an early period in the work, when the operative technic was uncertain, grafts coloring red with the phthalein were not infrequently encountered. It was natural to suppose that these, as manifesting a tissue reaction close to the normal, would be the ones to survive; and not until some thirty orange or red examples had been followed did the fact emerge that it was precisely those grafts which showed for days the orange color of an abnormal acidity that lived and healed into place. Whenever the implanted bit of skin, or a portion of it, repeatedly stained red during the early period after operation when it should have been reestablishing itself, that graft or portion was noted soon after to perish. Once this had been realized reasons for the color difference were not far to seek. Owing to poor fluid interchange the acid products of the cell metabolism of healthy grafts would tend

only toward the end of the 1st week, as the graft became established, did the hue gradually revert to the "normal."

The expectation had been that the blue erythrolitmin liberated in the frozen and thawed graft by death of the cells would be carried off little by little in consequence of the local fluid interchange, and that in consequence the color would become much lighter. But, as just mentioned, even the relatively slight amount of blue pigment liberated in the healthy graft persisted as such in it for several days. Erythrolitmin has an affinity for intercellular substances, which remain blue for months after the coloration has disappeared elsewhere (18); and it is highly colloidal, passing to and from the tissues with difficulty. In the light of these facts there is no need to invoke chemical alterations in the fabric of the several times frozen graft to explain its enduring blue color.

The observations demonstrate that the cells of grafts frozen and thawed repeatedly lose their semipermeability. That the change takes place everywhere and all at once may be doubted however: for not only do the grafts retain the aspect of life for many days but their color by transmitted light, prior to implantation after the injury, is ruddy here and there, showing that some of the erythrolitmin still persists in the red form. Only by reflected light is the hue a deep, clear blue.

Permeability of Skin Grafts for Carbon Dioxide.

The increased permeability of the damaged, avascular skin graft has been shown in another way, namely by submitting the body of a mouse carrying it and a healthy graft to an atmosphere of carbon dioxide. The gas penetrates the injured skin with immense rapidity, rendering it acid.

It was Lavoisier himself who first showed that carbon dioxide passes out of the intact skin of mammals; and his observations have been often repeated, with variations. When the skin is hyperemic and moist a not inconsiderable gaseous interchange may take place through it, as much as 4 per cent of the total CO_2 being eliminated in this way (19). To the present no experiments seem to have been made on the penetration of CO_2 from without. Skin grafts *in situ* are ideal for a study of the phenomenon since their avascular condition creates an opportunity for the gas to accumulate within them, as it cannot to any considerable degree within the normal integument.

For the purpose of the tests there has been utilized the funnel gas chamber devised for a study of the changes in pH occurring in raw tissue surfaces (20). The CO_2 was let in at *D* in the apparatus as figured in the paper referred to, and

under the conditions. Such heat regularly results in an eschar after 2 days, when applied to the shaved body of rats by way of a glass disc through which hot water circulates. But it was only after the lapse of about a week that the heated grafts became evidently necrotic. Until then they maintained the aspect of life, being pliable though definitely swollen and somewhat more opaque than control grafts on the other side of the same animal. They continued to be pallid, however, long after healthy grafts had begun to show the flush of a renewed circulation; and eventually they dried, remaining adherent over the advancing edge of the skin as it encroached on the defect beneath them.

If the appearance of the heated grafts was for a long time much like that of surviving tissue this was never true of their reaction. From the moment that they were sewn back in place they were always frankly alkaline to phenol red, as alkaline to appearance as the surrounding normal tissues and occasionally somewhat more so, being then of a more purple hue (Fig. 2).

Repeated rapid freezing and thawing, unlike heating, failed to render a graft more opaque than ordinary, and when first sewn in place it had precisely the appearance of the control graft on the other side of the same animal. Yet freezing and thawing is known to kill mammalian cells of many sorts; and I have repeatedly utilized it to destroy the cells of a transmissible chicken sarcoma without injury to the filterable agent responsible for the growth. It did cause eventual failure of the skin grafts. These retained their appearance of life for a week or 10 days, though, and often developed what appeared to be a surface union with the surrounding tissues. They never vascularized, however, but remaining pallid, and, becoming gradually thinner and parchment-like, they dried up. Unlike heated grafts they were never in the least edematous. From first to last the frozen and thawed skin stained red with phthalein, often a more purple-red than the animal.

It was, as has been stated, a part of the plan of the experiments to injure the implanted skin only to the extent necessary to insure a failure to "take." For, obviously, greater tissue changes would have lessened the chance that the graft would remain placed and in condition to take the stain, and have complicated the interpretation of the results. That the heating was close to the critical amount appears from the fact that a graft submitted to only 48°C. for 7 minutes

CO₂ did not alter the color for the good reason that the acid end of the range of phenol red had been attained. The control graft on the opposite side of the animal was *apricot orange* or *apricot buff* to begin with; and during the brief period in which the frozen and thawed tissue was running the gamut from purple red to orange it altered slightly, to *zinc orange*; but it obviously lagged in changing color as compared with the injured tissue and was still definitely less orange than the latter after 15 minutes, the maximum period of the observations (Fig. 4). On the other hand when once again exposed to the air it kept its unusually intense orange color long after the frozen and thawed skin had once again become *eugenia red*. This happened within about 15 minutes.

During the time that the grafts were undergoing these changes a slight but definite alteration was to be noted in the color of the body surface generally. It turned from red toward yellow, that is to say from *eugenia red* to *jasper red* in the case of animals approximating the first mentioned hue, and from *jasper red* to *carrot red* in some other individuals. There is, by the way, not a little variation in the surface hue of normal animals stained with phenol red, as the mention of these differing initial colors will attest. Variations in the normal pH of the blood, similar in magnitude to those here indicated by the phthalein have, of course, long been recognized to exist.

In order to determine whether the general change in color of the animals submitted to CO₂ was referable to absorption of this gas by way of the grafts or to a passage of it through the undisturbed skin everywhere, normal mice were shaved from fore legs to hind and 2 days later were exposed to pure CO₂. There ensued changes in the general hue identical with those just described. At the end of three-quarters of an hour they were no more marked than after 15 minutes. They were indicative of an apparent fall in pH from about 7.6 or 7.5 to 7.4 and 7.3 respectively. The rapidity with which the color reverted to the "normal" when the mouse was once again exposed to air was startling. Within 3 minutes the change had been completed. The surface acidosis described evidently resulted from a continuous passage of CO₂ through the skin, one not entirely compensated for locally, in the surface regions at least, by circulatory and respiratory readjustments. In a number of animals the rate and amplitude of the breathing were followed throughout the experiment. Changes accompanied the exposure to CO₂, and doubtless analyses of the expired air would have shown that no inconsiderable quantity of the gas, in addition to that resulting from body processes, was being given off through the lungs.

Water never condensed within the funnel chamber out of the CO₂; and the skin of the mice was as dry to the feel as ordinary. The findings certainly cannot be laid to the presence of an abnormal amount of moisture on the skin surface. Moistening the grafts led, as was to have been expected, to a more rapid penetration of CO₂ into them. To demonstrate this drops of distilled water were placed here and there on the skin and on the grafts before the gas was run in. They stood high and hemispherical, like dewdrops; and after various periods were

it escaped, in some part, through the other tube *E*, which had now been provided with a connection leading off to the floor. The mouse lay on top of two layers of rubber dam which in turn were spread upon an electrically heated pad. The animal had been stained with phenol red as usual and rendered quiet by the intraperitoneal injection of 0.15–0.2 cc. of 20 per cent urethane shortly before the observations were begun. These latter were only undertaken 2 days or more after the body surface of the animal had been shaved and the grafts placed, in order to rule out the possibility of any entrance of the CO₂ through surface abrasions. The hair had been removed from about the neck of the animal with a sodium sulfide solution; and a round hole in the rubber dam closing the gap at the margin of the funnel fitted the neck snugly. The edges of the dam were everywhere attached to the funnel by adhesive except below, where the rubber extended along the pad in a broad apron ending 6 to 10 cm. away from the head of the mouse. In order to rule out any possibility that the animal might inhale CO₂, a second piece of rubber dam was placed about its neck, over the first, to block off the gas chamber more completely; and the head was thrust into a small funnel through which a continuous gentle stream of air was blown against the nostrils. Tests with smoke after the experiments showed that some CO₂ escaped from beneath the funnel here and there, as well as through the proper outlet for it, but that none whatever could have been inhaled. A thermometer was introduced into the gas chamber through separate piercings in the layers of rubber dam, with the bulb lying between the groin of the mouse and the heating pad. The temperatures ranged between 36° and 38°C.

When an atmosphere consisting entirely of CO₂ was to be used the gas was led in from an ordinary Kipp generator after passage through a wash-bottle. The water in this latter never contained more than a trace of HCl, and there would seem to be no possibility that the effects on the skin surface of the mouse can have been due to another cause than carbon dioxide. The rapidity with which they disappeared when the surface was once again exposed to air also bespeaks the action of the gas. In some special experiments a mixture from a compression cylinder, containing 10.35 per cent CO₂ and approximately 20 per cent O₂, and 70 per cent N₂ was employed. Dr. C. A. L. Binger kindly determined the percentages.

When comparisons were to be carried out with the Ridgway color standards the stopper was briefly removed from the top of the gas chamber so that the inspection could be made without the intervention of a glass wall. Access to the body of the mouse could also be had in this way without changing the gas. To replace the latter with air a tube was thrust in and the chamber emptied almost instantaneously with the aid of the laboratory vacuum.

On running pure carbon dioxide into the chamber a change could be noted practically at once in the color of the frozen and thawed, avascular graft. It began to turn from *eugenia red* through *apricot orange* to a brilliant untempered *orange*, reaching this hue within 8 or 10 minutes (Fig. 4). Further exposure to

The Association of Tissue Acidosis and Edema.

Healthy skin grafts are regularly somewhat edematous during the first few days after implantation, at the time that is to say when the local reaction is acid as compared with that of the rest of the integument. A similar association of edema with tissue acidosis is not infrequent under other circumstances,—prevailing opinion and potentiometric determinations to the contrary notwithstanding. It can be observed to exist about surface abrasions in animals vitally stained with phenol red, as well as at other points of local inflammation, the phthalein in the swollen areas being orange as compared with the red of that in the normal skin nearby. And a rapid local development of edema and acidosis takes place when a tube through which water circulates at 50–52°C. is applied to the skin of an anesthetized rat stained with phenol red. Under such circumstances the edema and a change from red to orange of the phthalein contained in the region involved by it both become pronounced within 15 minutes.

In contrast to the swelling and acidity manifested by healthy grafts one sees in frozen and thawed grafts not the least edema, and a reaction which is definitely alkaline. The facts are not to be taken, however, as furnishing support to the hypothesis that acidosis determines edema. Edema develops, yes, in the living and acid skin graft, but it progressively disappears during the days immediately after the grafting, whereas the acidosis does not diminish. Furthermore edema is regularly to be met in grafts that have been injured by heat, although the reaction of the injured tissue is pronouncedly alkaline. In a succeeding paper observations are reported which would suggest that under the conditions of widespread and enduring tissue acidosis brought about with hydrochloric acid no important water retention occurs.

DISCUSSION.

The foregoing observations provide data on the conditions which prevail in skin grafts and determine their survival; but it is in a broader relation that they have principal claim to attention,—namely, in relation to the happenings within ischemic tissues as a class. Despite the general recognition that interference with the blood supply

removed. It was possible to do this with no other alteration of conditions within the chamber than were involved by the introduction from above of a long-handled forceps carrying a piece of filter paper to blot up the fluid. At times when the injured graft was becoming acid in consequence of the exposure to CO_2 but had not yet attained to the hue of *orange* a narrow circle of this hue could be briefly seen after the application of the filter paper, a circle corresponding in situation to the edge of the drop, the place at which the layer of water had been thinnest. When now the graft was reexposed to air the regions still moist turned purple first. Healthy grafts gave less outspoken findings; and only occasionally were slight differences of the general nature of those just described to be seen on the intact skin.

The experiments brought out a number of facts. Carbon dioxide penetrates the intact skin of the shaved mouse so rapidly as to cause some change in the surface hue of animals stained with vital red; it renders vigorous skin grafts somewhat more acid than they already are as the result of ischemia; and it penetrates injured grafts with an astonishing rapidity, rendering them pronouncedly acid. How acid the injured tissue can become is not yet certain, for no indicator other than phenol red has been employed.

Even in vigorous skin grafts there is much cell degeneration and death. The epithelium in particular is soon reduced to a thin layer of living cells (21). When the tissue has been injured experimentally the retrograde changes must be still greater; and one might think of the heated, or frozen and thawed, graft as a mere raw dead surface exposed to the air, did not its texture and the absence of seepage or drying belie the view. Doubtless the alkaline reaction of grafts thus injured is due in some part to an escape of carbon dioxide from them. The experiments involving exposure for a long time to a gas mixture containing 10.35 per cent CO_2 possess significance in this connection. Exposure to such a proportion of CO_2 , approximately twice that in alveolar air, leads to an alteration in the hue of injured grafts,—there is a change from *eugenia red* (or purpler) to *carrot red*; but the alteration is slight, as compared with the change to *apricot orange* promptly undergone by stained pieces of healthy skin when grafted in an atmosphere of air. Exposure to 10.35 per cent CO_2 does not result in any definite color change in the mouse's body surface generally, and leads to only dubious ones in healthy grafts already *in situ*.

autolyzing in the mass *in vitro*; and perhaps they can be safely applied to large masses autolyzing *in vivo*. The interchange of material with the body round about will have little significance for the immediate fate of large infarcts and accumulations of pus despite the unusual permeability of dead tissue. But when the necrotic mass is small the factor of interchange assumes great importance, as the experiments with the skin grafts show. The occurrences subsequent to the injury of small cell aggregates cannot be explained on the basis that a local accumulation of acids determines autolysis or atrophy (23), for the sufficient reason that the local reaction will tend toward alkalinity rather than toward the acidity of vigorous tissue surviving an ischemia. It follows that the chemical changes which take place in small necroses must differ in some respects from those occurring in large masses of dead tissue. A single set of generalizations as concerns autolytic processes will not cover both instances.

While the life processes of vigorous tissue suddenly rendered bloodless act to create a milieu that would seem prejudicial to survival, the alterations that take place in injured tissue would appear superficially to favor this event. To judge from the observations on skin the lessened cell activity consequent upon injury results in a smaller accumulation of acid material,—save for that referable to the trauma itself (as in muscle); and the increased permeability of the damaged tissue results in a more rapid escape from it of carbon dioxide and of the other substances causing acidity. But needless to say the rough correspondence thus brought about between the reaction of the injured graft and the normal tissue surrounding it is a superficial phenomenon, not the sign of a good state of affairs but of one which masks profound cytological derangements.

The eventual drying of grafts which fail to "take" is not due to any failure to obtain fluid from underneath, as the experiments have shown. Rather must one think of it as consequent on an abnormal loss of fluid from the surface, itself a manifestation of the increased permeability which can be demonstrated by exposure of the tissue to carbon dioxide. There is good reason to suppose that the reaction of the tissue involved in skin lesions often deviates from the normal owing to the influence of the factors dealt with in the present work.

The observation that edema and tissue acidosis sometimes coexist

of a living tissue results in the local formation of acid substances the corollary that ischemic tissues must endure for some time in an acid milieu if they are to survive seems not to have had the attention that it merits. As the present work shows, the more vigorous the cells the more acid do they render the tissue when its blood supply is interfered with; and the tissue survives despite this acidity. Skin has a relatively slight metabolic activity, and some part of the carbon dioxide accumulating in avascular grafts of it must escape to the air or into the body of the animal, while a continuous, if slow, fluid interchange with the neighboring tissues acts also to reduce the local acidity. Yet notably acid the tissue is, nevertheless. This being the case what must one suppose the reaction to be within a leg severed and reunited without suture of the vessels (Halsted), or in an arm surviving despite a clot in the axillary artery? Can one doubt the development of a more pronounced acidity in these ischemic members?

That cells of some sorts will survive and proliferate *in vitro* in a frankly acid medium is a fact sufficiently attested (22). But the occurrence of proliferation renders the case somewhat different from that of tissue surviving within the body under acid conditions. For it might well be that the individual elements of a culture tolerate the condition of acidity but poorly, and that the strain survives only because its elements continue to divide, furnishing fresh entities more rapidly than the injurious medium kills them off. An analogy to this state of affairs is to be found in the growth of certain tumors composed of cells surviving for so short a time that retrogression of the mass would inevitably ensue were not the rate of wastage more than outstripped by that of proliferation. The condition is one familiar to every student of neoplasms. It is often plainly evident when "Chicken Tumor I,"—a transmissible sarcoma,—is cultivated *in vitro*.

No attempt has been made in the present work to determine more than approximately the degree of acidity developing in the grafted skin of the mouse. Manifestly the knowledge could have only a special interest; for one would expect a much greater acidity to develop in tissues of high metabolic activity. To determine the relative reaction within ischemic portions, living and dead, of organs which appear to be frankly acid under normal conditions,—the liver and pancreas for example,—will be an interesting task for the future.

Current generalizations on the changes which take place within tissues dying in the body are largely based upon studies of material

The observations here set forth may, perhaps, be thought of as the first steps in an analysis of the physical factors which act to determine the fate of engrafted tissues. It is habit to suppose that this fate depends on the ability to survive temporary ischemia, on absence of infection, prompt vascularization and, in the case of iso-grafts, on a tolerance by the host of the strange tissue, and by the graft of its alien surroundings. So of course it does. But the first requisite for survival, namely the ability to survive ischemia is directly referable to physical conditions within the graft, as is also, doubtless, the development of the vasculature that will eventually rescue the tissue from its precarious state.

Gesell has recently brought forward (27) a theory of respiratory control based on the assumption that changes in the hydrogen ion concentration of the respiratory center are the responsible influence rather than changes within the blood. The happenings within skin grafts furnish a suggestive analogue to what goes on within the center according to the view of this author.

SUMMARY.

By means of vital staining with indicators a study has been made of the changes in reaction and in certain other attributes of a tissue abruptly rendered ischemic. Grafts of mouse skin have been employed as test material. It has been found that almost at once after implantation vigorous grafts become notably acid as compared with the normal skin and that they survive and "take" despite the acid condition, which remains at a maximum for several days. Weak or injured grafts on the contrary tend to be as alkaline as their surroundings, if not more so. Through experiments directed to the purpose reasons for this difference have been found in the lessened metabolic activities of the cells of the injured skin, and in an increased permeability which leads to a generalized suffusion of the damaged tissue with the alkaline lymph and an abnormally rapid escape of carbon dioxide from it. The influence of these factors to determine the reaction of tissues dying within the body has not been sufficiently taken into account in considering the chemical changes that occur after cell death, and some revision of current views regarding these as they affect small necrotic masses would seem called for.

would at first sight seem difficult to reconcile with the fact that edema fluids as obtained for potentiometric examination are regularly alkaline (24). But the contradiction is merely apparent. Local acidosis and edema occur together only when the fluid accumulation is not very pronounced and the metabolic activities of the tissue are either abnormally heightened,—as during inflammation,—or are taking place under conditions which permit of an accumulation of acid products,—as in skin grafts. There is little doubt that the development of edema can act to maintain alkalinity, the profuse alkaline fluid deriving from the blood having effect to drown out, so to speak, what might otherwise be a local acidosis. I have never found a very pronounced inflammatory edema that did not yield a fluid alkaline to phenol red, although inflammation as such conduces to local acidosis. And Henning (25) who injected $N/10$ HCl into the leg muscles of guinea pigs, observed that the initial acidity was supplanted after 24 hours by a pronounced local edema and alkalinity. It is conceivable that sometimes during the development of an inflammatory edema there may be such an escape of blood protein into the tissues as will suffice to influence phenol red, with result in a greater alteration in the color of the phthalein than the actual acidosis would warrant. No such happening can be invoked to explain the case of edematous skin grafts, however.

Some comment is necessary on the difference between the hue of the skin surface of the vitally stained mouse, as seen by reflected light, and the color of the corium—the skin component principally stained with phenol red—when viewed by transmitted light. The purple red of the former corresponds with pH 7.5 to 7.6, as ascertained by the wedge method described in the present paper; whereas the connective tissue of the corium, when examined separately under oil, has the yellow-pink of about pH 7.2 (26). The reasons for this difference—one which persists when the blood vessels have been flushed out—are largely to be found in the differing optical conditions, but also in some part in the suffusion of the tissue with an alkaline lymph, heavily charged with phthalein, and in consequence ruddy. When a flap of the oiled skin surface of a deeply stained mouse is compressed between slides so that the interstitial fluid is forced out of it for a moment its color is altered from yellowish red to orange.

EXPLANATION OF PLATE 32.

FIGS. 1, 2, and 3. Right and left sides of a mouse, to show the course of the vital staining of grafts with phenol red. Period: any time during the first 3 days after implantation.

FIG. 1. The staining 15 minutes after an intraperitoneal injection of the indicator. The healthy graft shows no color as yet, whereas the one that was frozen and thawed has become pink. The animal is already deeply colored.

FIG. 2. 45 to 60 minutes after the injection. The grafts are now intensely stained, the hue of the healthy one being indicative of a condition of relative acidity, whereas that which has been injured would seem to be slightly more alkaline than the normal tissue round about, judging from its color. The mouse itself is beginning to decolorize.

FIG. 3. 3 hours after the injection. The animal is now completely decolorized but there is still much phenol red within the grafts and the same differences are visible in them as before.

FIG. 4. Effect on the phenol red coloration of exposure of the body surface to an atmosphere of carbon dioxide for 15 minutes. The injured graft is now no longer alkaline but frankly acid, as evidenced by its hue; and the healthy graft while slightly more acid than ordinary is not as acid as the injured graft. The color of the body surface generally is indicative of a slight change in the direction of acidity.

FIG. 5. Right and left sides of a mouse stained with erythrolitmin, to show the alterations in color of engrafted skin and of skin that has been purposely damaged as well as engrafted. The healthy graft is slightly bluer than the body surface generally, a fact which attests to some injury to its cells with a loss of the normal semipermeability; and the graft which was frozen and thawed is deep blue,—evidence that the cells have become freely permeable to the alkaline body fluids.

Carbon dioxide penetrates so readily into the living skin as to cause some local increase in the hydrogen ion concentration within cutaneous regions exposed to an atmosphere of it, even when the local circulation and the ventilation by way of the lungs have not been interfered with. It penetrates injured skin with especially great ease.

Tissue acidosis and edema not infrequently occur together; but no relationship between them of cause and effect has been made out.

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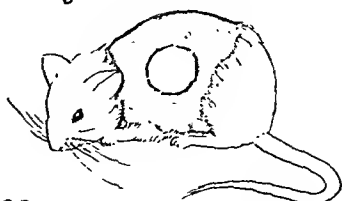
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Healthy grafts

Injured grafts



1



15 minutes



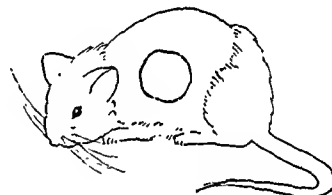
2



45-60 minutes



3



180 minutes



4



Exposed to CO₂



5



Stained with erythrolitmin

the practice of which involves distortion from the conditions of life. Mention should be made, however, of the interesting experiments of Stieglitz on the reaction of the living kidney as affected by the administration of acid or alkali (2).

Method.

For the present work rats vitally stained with phthaleins were employed; and the living tissues were examined under circumstances which would seem to exclude the ordinary errors of interpretation.

A pair of male rats, from the same stock and of the same age and weight, preferably not full grown, were used in each experiment. They were given no food on the morning of it. The hair was removed from the chest, abdomen, and sides a day or two beforehand in order that any inflammation due to the shaving might have cleared up. On beginning the experiment each animal was given 20 per cent urethane (3) subcutaneously into the back of the neck, in the proportion of 1.1 cc. for every 150 gm. weight, and some minutes later the phthalein into the peritoneal cavity. After $\frac{1}{2}$ hour or more, when the general staining was pronounced, the animals, by now unconscious, were submerged to the muzzle in warm paraffin oil in glass dishes placed side by side under identical conditions of lighting, and after brief preliminary observations the injection of acid or alkali into a jugular vein was begun.

The rectangular Pyrex dishes had a layer of solid paraffin covering the bottom, with a depression for the urine, and they held sufficient liquid paraffin oil—cleansed, and inert to phthalein—to cover the rats. The oil was kept warm by the heat generated when a current was run through coiled "Nichrome" wires surrounded by oil and encased in glass tubing bent to conform to the shape of the dish (Fig. 1). The heating was regulated by means of a rheostat. To distribute it evenly the oil was stirred at intervals with a spatula. The temperature in the two dishes was maintained between 38° and 41°, as nearly the same in both as possible. The legs of the rats were kept in extension by means of long pins thrust through the skin of the paws and into the paraffin bed, while their muzzles were held above the surface of the oil by a thread carried through the skin of the nose and tied about a knitting needle placed across the dish from side to side. Before the oil was poured on, a fine cannula was inserted into a jugular vein of one of the animals, and connected with the apparatus for the injection of the acid or alkaline solution. The other animal served as control. A special arrangement was devised

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- (3) The influence of urethane upon the blood reaction of rats is known, thanks to the work of Hawkins (Hawkins, J. A., and Murphy, Jas. B., *J. Exp. Med.*, 1925, xlii, 609). For 3 hours or more after injection it causes little change but then there ensues an alkalosis. Our experiments were completed within the initial 3 hours.

THE RELATIVE REACTION WITHIN LIVING MAMMALIAN TISSUES.

VII. THE INFLUENCE OF CHANGES IN THE REACTION OF THE BLOOD UPON THE REACTION OF THE TISSUES.

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The blood is all things to all tissues, and in consequence it can scarcely be expected to manifest the peculiarities of any one of them. Special characters of its own it must have to serve so widely as it does. These elementary principles are often overlooked by those who would speculate upon hydrogen ion concentrations within the body; and one finds not infrequent a disposition to suppose that the tissues and blood must have the same reaction. In several previous papers from this laboratory evidence has been produced that the hydrogen ion concentration within the individual organs differs somewhat (1). In this respect the tissues would appear to be related to the blood as to a sort of physiological base line, from which they stand off at various distances along the ordinate of hydrogen ion concentration. When one produces alterations in the level of the base line, as in the experiments now to be described, the position of many tissues along the ordinate alters also; they become relatively more or less acid or alkaline in rough correspondence with what happens in the blood. Other tissues however, as we shall show, retain their apparent pH unchanged even when the amount of acid or alkali introduced into the animal is sufficient to cause death.

The accumulated data with regard to the various offscourings of the organs as they appear in the blood and the excreta have furnished material for many inferences upon tissue states. But these have remained inferences at best, subject to modification with each new parcel of facts discovered. There have been few attempts to study the reaction of the tissues directly save by quantitative methods

to deliver the injection fluid at body temperature (Fig. 1). The nozzle of a calibrated burette (*F*) which served as a reservoir was connected with a drop gauge (*E*) and this in turn with a long rubber tube coiled in a bath containing hot water at a constant temperature (*A*). The tube passed out through a side arm from the bath, and was encased, as far as the oil bath, in a much larger tube (*B*) containing some of the same warmed water that was in the bath. In order to ensure a circulation in this elongate jacket its further, stoppered end was equipped with an exit pipe (*D*) for the water, in addition to the opening for the rubber tube carrying the injection fluid (*H*). The water was kept flowing through the jacket and back into the bath by the introduction of a slow stream of illuminating gas through a side arm (*C*) of the exit pipe. The passage of bubbles of gas along the pipe (*J*) acted to move water back to the water bath. The gas was delivered separately to be burnt beneath this latter. The water of condensation from the gas collected in the bottle (*M*). For the idea embodied in the apparatus we are indebted to Dr. D. R. Drury.

The tube carrying the solution to be injected now passed beneath the surface of the oil and laterally along the dish,—where its temperature was measured with a thermometer let into the system,—and so to the animal. Near the cannula there was a side arm,—which is not figured,—to permit an adjustment of the pressure immediately after connection with the jugular had been made. Otherwise, when the clamp was taken from the vessel prior to the injection there was sometimes a sudden gush of fluid into the blood with death at once. To prevent blood from passing back into the cannula the latter was turned at an angle to the course of the vein so that its wall would act as a valve across the opening in the glass.

The intensity of the staining of paired rats injected with an equal dose of phthalein was frequently found to differ somewhat; and when phenol red had been used the surface hue often differed slightly as well, variations from *eugenia red* to *jasper red* (4) being observed. The differences in extravascular pH, indicated by these differences in hue, are no greater than the long recognized individual differences in pH of the normal blood. In order to minimize such errors as might be introduced by them when the tissues of the injected and the control rat were compared, several individuals of the same size were often given phenol red at one time and from these the two were selected that most closely corresponded in the character of the staining.

As many of the tissues were examined *in situ* as possible. Since skilled assistance was available the examinations could be made very rapidly. The color of the body surface generally, and of the paws, ears, and mucous membranes, gave

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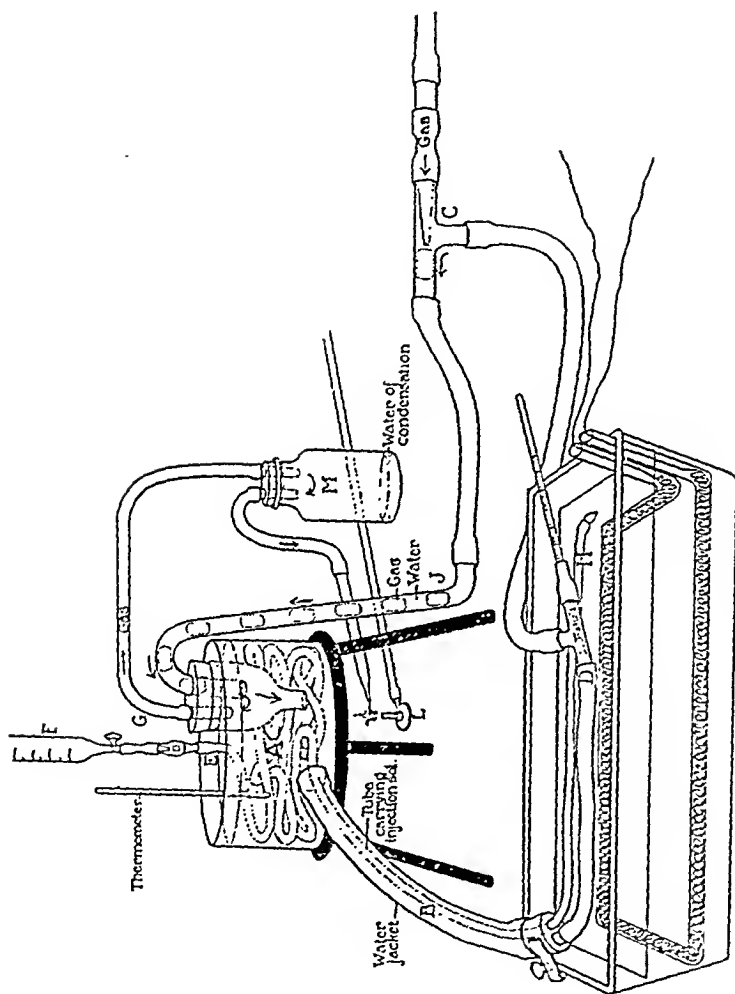


FIG. 1.

The phthaleins employed were cresol red, phenol red, and brom cresol purple. They were employed in watery solution isotonic with the blood and at pH 7.4 except where otherwise stated. The staining was at a maximum when the injection of acid or alkali was begun, practically all the phthalein having been absorbed from the peritoneal cavity. Brom thymol blue could not be used since it is toxic and gives false indications *in vivo* (6). Nothing has been found to date despite much search in our laboratory that will take its place in covering the zone between the ranges of phenol red and brom cresol purple,—a zone in which the reaction of many of the tissues would seem to lie.

Changes in the Coloration Result from the Injections.

In some preliminary observations, to determine the general effects of acid and alkali upon the hues of the stained tissues, relatively concentrated solutions of sodium carbonate and of hydrochloric acid were run into the blood stream.

These had the strengths employed by MacNider (7) in his studies upon kidney function, that is to say they were equimolecular with 1.5 per cent NaCl and, in the case of carbonate, 3 per cent NaCl (concentrations slightly less than $N/2$ and $N/1$); and hence they were notably hypertonic. To lessen the likelihood of anhydremia from this cause, with "outlying acidosis" as a sequel (8), several cc. of warm water was given to the animal by stomach tube some minutes prior to the injection. The acid or alkali was run in as fast as possible, which was after all rather slowly. Tests showed that about 4 cc. of $N/1$ Na_2CO_3 could be introduced into a rat of 170 gm. in the course of an hour, with the animal still in excellent condition, but if the rate was increased a very little, or a few drops were inadvertently run in together after the injection had proceeded for some time death occurred immediately. Since $N/2$ HCl greatly damages the red cells $N/4$ HCl was early substituted for it. More than 5 cc. of the latter solution, given during the course of half an hour, was tolerated by a rat of 140 gm. The patching characteristic of an "outlying acidosis" was never noticed.

Shortly after the administration of acid was begun the secretion of urine stopped, as in the case of MacNider's animals; whereas that of the alkali was attended by a profuse diuresis. The injections were pushed to the lethal point, but from time to time while the condition of the animal was still good, the color of bits of its vitally stained organs were compared with those from the control by the technic above described. It was soon found that there exists a group of tissues,—connective tissue, tendons, fascia, and cartilage,—which undergo marked alterations in hydrogen ion concentration, as indi-

direct indications of the reaction of lymph, connective tissue, and cartilage. By a bloodless incision the tendon could be exposed, as too the lymph node of the groin, and the voluntary muscle and the cartilages of the thorax. But the colors in general were most truly seen when the tissues were viewed by transmitted light. This was possible without disturbance, in the case of the connective tissue of a skin fold, and of the cartilage of the ear. Small bits of the other organs were snipped off, and, still surrounded by oil, were examined between mica slips, those from the control and the experimental animal being studied side by side. Glass slides could not be used because of the alkali deriving from them (5). A special plan was followed in taking the specimens to rule out the possibility that the differences noted were due to supravital changes. Thus, when an animal had been injected with acid the bit from it was removed for inspection only after that from the control had been secured,—since the bit longest out of the body would tend to be the more acid as result of the longer asphyxia from interruption of the circulation. If now under these circumstances the tissue fragment just removed from the injected rat appeared more acid than that from the control there was good reason to suppose that a real difference in reaction had existed within the body. Sometimes, to obtain slices of the parenchymal organs, Valentine knives were employed, a different one for each animal, and the cutting was done under oil, on a paraffin base. Bleeding from severed vessels was prevented with mosquito forceps; and cleansed and dried instruments were employed with each specimen. Special care was necessary to avoid contamination with the blood, which, having received directly a greater or less quantity of the acid or alkaline solution, was, of course, altered thereby. In the cases of the parenchymal organs such contamination inevitably occurred, but fortunately it was the tissues of just these organs that remained uninfluenced in reaction by the injections. Cartilage, connective tissue, and tendon could readily be obtained blood-free. It is possible to procure preparations containing all three of these tissues and bone as well from the tip of the tail of the young animal (65 to 90 gm.). This is snipped off with scissors, gently pressed between folds of washed gauze to drive out the blood, and the bony core is rapidly extracted under oil and mounted between mica slides for inspection by transmitted light. By properly timing the procedures one can compare the experimental specimen directly with that from a control treated in precisely the same way and after the same brief interval of separation from the body. Bone and voluntary muscle were not satisfactorily stained under the circumstances of the experiments,—which usually involved a considerable diuresis with color loss,—nor was the epidermis stained nor the splenic substance. A study of the stomach, intestines, and lungs was not made. To all practical purposes, then, the observations were limited to the superficial color, to the hue of the tissue of the ears, *in situ* or on dissection, to connective tissue and cartilage as such, tendon, liver, pancreas, kidney, and superficial lymph nodes. When dissolved in fat the phthaleins no longer act as indicators, though they do in the adipose tissue itself.

having the orange-pink of an apparent pH of about 7.2; and the more ruddy surface hue is to be ascribed, in part to the optical conditions and in other part to a staining of the alkaline interstitial fluids (9). When cresol red has been injected the oiled surface of the stained rat appears yellow, slightly tinged with brown, and all the stained tissues are a brilliant yellow. Several experiments were made to determine whether the color could not be altered to the rose-purple which is characteristic of a pH of 7.8 or more, under controlled conditions.

In an initial test a Na_2CO_3 solution isotonic with 1.5 per cent NaCl was gradually injected into a rat of 110 gm. The animal died when 9.0 cc. had been run in. The urine was orange-red to begin with but later purple red; yet none of the stained organs changed from the usual yellow hue. In the course of further observations, with $N/1$ sodium carbonate, the blood changed from orange-red toward purple, and the surface color from yellow to rose-pink, after 3.7 cc. of the solution had been run into an animal of 170 gm. stained by the injection of 1.7 cc. of 4 per cent cresol red solution. The nose, lips, and ears became light red; yet the tissues generally appeared of the same clear yellow as in the control, when the blood and intercellular fluid were rapidly pressed from them. A rat of 250 gm. given 10.4 cc. of $N/6.35$ sodium carbonate became ruddy orange, and its urine much more purple than that of the control. The ears *in situ* appeared ruddy and so too did a skin flap dissected up and viewed by transmitted light. But a wedge-shaped piece of the ear, rapidly excised and compressed to drive out the fluid, was as yellow as the ear of the control; and so too with all of the other tissues examined, connective tissue, cartilage, tendon, voluntary muscle, liver, lymph nodes, kidney, and pancreas. Many of these looked somewhat ruddy until pressed upon to expel the stained fluid associated with them.

It was obvious from these experiments that only the blood and intercellular fluids could be altered sufficiently in reaction by the injection of alkali to come within the range of cresol red. This would have meant a considerable change, granting,—what has not yet been proven,—that cresol red when in the body serves to indicate the pH as accurately as it does *in vitro*. For a rose-purple hue of the tissues could scarcely be expected short of pH 7.8 or 8.0; and the most alkaline of them, the connective tissue, exhibits under ordinary circumstances when stained with phenol red,—an indicator well tested in this relation (10),—the hue indicative of an approximate pH of 7.2.

cated by the phthaleins, when acid or alkali is given. The hue of certain other tissues, parenchymal in make-up,—those of the liver, pancreas, and lymph nodes,—remains practically unchanged so long as life lasts. The tissue which responds soonest to the alteration in the reaction of blood is the connective tissue.

Saline Solution Fails to Influence the Tissue Reaction.

More extensive experiments were now undertaken with solutions isotonic with the blood ($N/6.35$ solutions). Large amounts, 15 cc. or more, were tolerated by rats of 150 gm.; and there was diuresis after the acid as well as after alkali. The question arose of how far the observed alterations in the tissue colors were referable to the amount of fluid introduced, merely as such. To control this factor some injections were made of 0.92 per cent NaCl. It was found that 20 cc., run into 150 gm. rats at the rate of the acid or alkali, caused not the slightest change in the hue of the tissues stained with phenol red except the fading incident to rapid elimination of the indicator. A like result followed the injection into a 110 gm. rat of 15 cc. of isotonic dextrose solution (5.4 per cent Merck's dextrose in twice distilled water).

The findings with hypertonic solutions of acid and alkali did not differ essentially from those with the isotonic solutions save that in the case of the former the phthalein was retained longer in the body; so it will be unnecessary to discriminate between the two sets of experiments in detailing the results.

Effects of Alkali on the Coloration with Cresol Red.

The surface hue of a living rabbit stained with phenol red and viewed under oil by reflected light is indicative of a reaction between pH 7.4 and pH 7.6, as can be shown by comparing it, through an Autenrieth wedge containing water, with the hue obtained by placing on the surface of a shaved but unstained control, one or another of a series of similar wedges filled with buffer solutions of appropriate pH, stained with phenol red. The surface color of urethanized and stained rats is indicative of the same slight degree of alkalinity. The subcutaneous tissue as such appears relatively acid by transmitted light,

the hue of their sheaths was changed from pinky yellow to old rose,—which one would expect from their character as connective tissue; and the walls of the large arteries were old rose. The lymph was much purpler than in the controls.

The changes observed in animals given strong alkali were duplicated when it was administered in N/6.35 solution. The isotonic carbonate solution was especially well tolerated, and we have injected 6 cc. into an animal of 65 gm. during the course of 1½ hours without causing death, and 27½ cc. into a 200 gm. animal in about the same time. Whenever there was an initial color difference in the pair of rats we regularly injected that individual which had the lighter, and sometimes the less alkaline, hue,—*jasper red* for example as compared with *eugenia red*. The changes brought about by the alkali were the more remarkable under such circumstances. As the injection progressed one saw the rat which was originally the lighter with perhaps a slightly yellow cast to the surface red, become relatively dark and purple, and those of its tissues turning toward purple appeared more markedly stained than was the case in the control, although they really had less of the phthalein in them—a fact demonstrated by applying alkali to bits of both *in vitro*. The tissues of the injected animal which did not alter from the ordinary hue, remaining yellow, were a paler yellow than those of the control because of a more rapid loss of phthalein by elimination of it from the body.

Situation of the Phthaleins.

Changes in the color of the blood can be ruled out as a cause for the alterations in the tissue hue, save in the case of especially vascular organs (ears, nose, mouth, and tongue) viewed *in situ*; for the prevailing color on staining with phenol red and other phthaleins is due to an extravascular penetration of the dye (12). It might be urged however that changes in the reaction of the interstitial fluids, secondary to those taking place in the blood, will suffice to explain the observed color phenomena. This is not the case. True, the suffusion of the matrix tissues with purple interstitial fluid tends to intensify their color. But the phthalein has a tendency to become fixed on the tissue components themselves (13). A bit of connective tissue or tendon removed from a rat stained *in vivo*, frayed with the knife, and examined under the high power, will regularly show a brilliant homogeneous staining of even its finest filaments. So too with the thinnest edge of a sliver of cartilage. The cells of such organs as stain yellow with phenol red, as if acid, are yellow even after alkali has been administered despite the presence of a purple red interstitial fluid, that is to say one which is relatively alkaline. Whether the cells of the matrix tissues as well as the ground substance become stained is not certain. It is safest certainly, for the present, to look upon the phthalein findings as expressive of the reaction within tissues, as distinct from that within cells (14).

The results obtained with phenol red were consistent. They showed not only that altering the reaction of the blood toward alkalinity

Effects of Alkali on the Coloration with Phenol Red.

The color of the shaved and oiled body surface of healthy animals injected with phenol red and viewed by reflected light corresponds, as has been stated, to that associated with a pH of 7.4 to 7.6 under controlled conditions. Many of the organs, notably the parenchymal ones, are stained clear yellow, indicating that their reaction lies beyond the range of the phthalein on the acid side; but two of the matrix tissues,—the connective tissue and tendon,—exhibit shades of pinky yellow which point to the presence of a relative alkalinity within them, the connective tissue appearing to be at about pH 7.2, and the tendons at, or slightly above, pH 7.0 (11). If the introduction of alkali into the blood alters the reaction of these two tissues one would expect the change to be demonstrable with phenol red since their apparent normal reaction lies well within the range of this phthalein. Such is the actual case.

In preliminary experiments hypertonic solutions of sodium carbonate were employed. Shortly after the alkali had begun to run in the rats were noted to be purpler than the controls, and soon the general hue was a brilliant rose-purple, especially pronounced on the nose, mucous membranes, and ears. The blood coursing through the latter was seen to be intensely purple as compared with red in the controls. The urine, now voided in great quantity, was no longer stained red but purple. When the alkalosis had become pronounced examination showed that both the connective tissue and tendons had altered from pinky yellow to old rose; and the cartilage was affected as well, being now ruddy orange instead of the usual orange-yellow. Some young animals of 50 to 80 gm., in which the injection was pushed to the limit compatible with life, had flat bones of the skull that appeared pinker in the growing portions than those of the controls: but owing to difficulties of the dissection this difference cannot be stressed. The differences observed in tissues of the "core" of the tails of young animals were clear-cut, and leave no doubt that the reaction of the connective tissue, tendon, and cartilage had been altered *in vivo*. The brief ischemia incident to the examination could not have killed these hardy matrix tissues, of course. Wedges cut rapidly from the ears and pressed to force out the ruddy interstitial fluid were orange in the case of the injected animal, yellow in the control, as would follow from the fact that they consisted mainly of cartilage.

The pancreas, liver, lymph nodes, and kidney cortex were always of the same brilliant yellow as in the control even when the animal was moribund from the large amount of alkali injected. In one case the Malpighian bodies of the spleen were also observed to be yellow. The peripheral nerves were unstained but

contrary a greater elimination by the experimental animals. The alkali injection regularly resulted in profuse diuresis, with a purple urine; but the urine of the controls was purple also, that is to say alkaline, probably because of the slight excess of alkali introduced with the indicator. By injecting the control animals with sodium carbonate, at a time when they had come to appear much less purple than the experimental ones they could be brought to the same, or a deeper hue.

The blood and lymph (as pressed from the superficial lymph nodes) both appeared blue-purple prior to any administration of carbonate; and they did not become more purple after it. The fact carries implications of importance. When really at pH 7.4 to 7.5,—the reaction of rat blood (19),—brom cresol purple is so largely dissociated that further alkalization fails to result in an increase in the purple hue appreciable to the naked eye under test-tube conditions. It follows that no change in the color of blood stained with the phthalein should occur upon alkalization *in vivo* if the circulating indicator has still the range characterizing it under controlled conditions *in vitro*. And no change does occur either in blood or lymph. Yet there is an alteration in the color of the body surface, which becomes more purple. This can only be attributed to an alteration in the hue of the subcutaneous tissues,—the actual event as direct inspection shows. These tissues, less alkaline than the blood, as judged by their hue with phenol red, must be less alkaline too with brom cresol purple; for otherwise the injection of sodium carbonate would not cause their color to intensify. The results with the two indicators are consistent in the demonstration of this difference in relative reaction.

Not only does the connective tissue of the animal injected with carbonate become more purple but so too do the tendons and cartilage. When a segment of the ear, which consists mainly of the last of the tissues mentioned, is excised and compressed between mica surfaces it can be seen to be far more purple than the control segment; whereas the almost blood-free fluid extruded from it and protected by oil has the same hue in both cases. The core of the tip of the tail of the injected rat also shows a more purple cartilage, and the costochondral articulations are a deeper purple. The bones stain poorly, and no satisfactory color comparison is to be had with them. In contrast to the ease with which the hue of most of the matrix tissues is altered that of the parenchymal organs evinces no change. The blue-green or yellow-green color of the lymph nodes, liver, and pancreas remains the same even when so much alkali had been introduced that the animal is in a moribund condition.

Valentine knife sections of the kidney cortex of our alkalinized animals were purple to the eye as compared with the yellow-green of the control tissues; and under the microscope one could see that the tubules contained a far more intensely purple urine and that a great many of them were colored purply blue. The differences will not be gone into however since they were the product of especially complicated conditions. An account of them will be reserved for a later paper from this laboratory.

resulted in changes in the reaction of some of the tissues, but that the ones evidently affected were those of which this might have been predicted owing to the ease with which they are penetrated by substances escaping from the blood stream (15) and to the further circumstance that ordinarily their apparent reaction, as indicated by the color they assume with phenol red, lies well within the range of this phthalein. Of the tissues with reactions lying beyond the range of phenol red,—on the acid side of it,—only one was so influenced by the alkali as to alter in hue. This tissue, the cartilage, would appear to be only slightly more acid under ordinary conditions than are connective tissue and tendon, occupying in this respect a middle place between them and the parenchymal organs (16). For all that is known to the contrary the reaction of these latter organs might have been changed by the alkali without bringing them within the range of phenol red. To test for such an occurrence, as well as to obtain a check on the general findings, brom cresol purple was now employed.

Effects of Alkali on the Coloration with Brom Cresol Purple.

Brom cresol purple is not stable in a 4 per cent solution brought to pH 7.4 but undergoes a rapid chemical change (17) which leaves it something of an indicator still but with a different range and inferior color values. The deterioration can be prevented in 1 per cent solutions by a special mode of preparation (18), but at 4 per cent it begins within a few minutes at most after the titration, as a change from purple to orange, starting at one point as a rule and rapidly spreading throughout the fluid. It does not take place when there has been a slight overalkalinization, and resort was had to this procedure in order to obtain stable preparations for injection. 400 mg. of brom cresol purple was taken up in 1.6 cc. of $N/1$ NaOH and diluted to 10 cc. with distilled water. Had 1.48 $N/1$ been used instead the solution would have been at pH 7.4. The rats tolerated well the intraperitoneal injection of the phthalein treated thus, when given in the proportion of 1.6 cc. for every 150 gm. of animal. Test observations on the color of the indicator diffusing into buffer solutions from pieces of the vitally stained tissues proved that it had undergone none of the deterioration just described.

The rats stained with brom cresol purple and injected with sodium carbonate, either in isotonic or hypertonic solution (54.6 gm. to a liter, equimolecular that is to say with 3 per cent NaCl), showed striking alterations in the surface hue. They became much more purple, *dull blue-violet*, in Ridgway's nomenclature, as compared with the *dull violet-blue* of uninjected animals. The phenomenon could not be accounted for by a retention of the dye in the tissues at a time when the controls had begun to decolorize, since no such retention occurred but on the

Skin flaps dissected up in such wise as not to disturb the circulation markedly, appeared pale greenish blue in contrast with the intense purply blue of the control, when viewed under oil by transmitted light. The ears were purply green, not a frank purple as in the uninjected animal; and when segments were cut and rapidly compressed between mica slides to drive out fluid, the one appeared brilliant green and the other a frank purple. The extruded fluid, protected by oil, was in both cases purply blue. No better evidence of the independence of the tissue color could have been desired. Valentine knife sections of the ear of the experimental animal showed a blue-green, or even yellow-green, connective tissue, as compared with the deep blue of the control, and a light green, or greenish blue, cartilage, that of the control being a deep purply blue. The tendons, purply blue in the latter animal, were a paler blue or else green in the rat given acid, and a like contrast was to be observed in the wall of the femoral artery. These differences cannot be laid to a greater decolorization as result of the injection; for the application of alkali to pieces of the tissue showed that those from the injected animal contained as much and often more of the phthalein.

The color of the parenchymal organs appeared practically uninfluenced by the acid, save in the case of the kidney. The renal cortex, as viewed in the gross, had undergone an obvious change toward yellow, though the microscope showed much of the difference to be due to a more acid secretion, the fluid within the tubules being green, not purple as in the control. There was, in addition, some yellowing of the tubular epithelium. The pancreas, though, and the lymph nodes, never altered from the ordinary yellow-green staining, nor did the liver from its characteristic blue-green except in animals moribund from the acidosis, when it was noted to have changed very slightly toward yellow. The change may conceivably have been due to alterations in the reaction of the bile which was deeply stained (21).

It will be seen that the findings corroborated those with alkali in pointing to the existence of a group of tissues readily influenced in reaction by the introduction of acid or alkali into the blood stream, and of another group not susceptible to such change. And, as in the experiments with alkali, brom cresol purple gave information confirming and extending that obtained with phenol red.

The Changes Resulting from Injections of Lactic Acid.

Some subsidiary tests were made on the effects of lactic acid in isotonic solution (0.314 N) upon animals stained with phenol red. The injection fluid was tolerated in large amounts, 14.5 cc. failing to cause the death of a rat of 126 gm. into which it was run in the course of only a little over an hour; and unless a flow into the jugular was

These observations concluded the series on the effects of injected alkali on the coloration of tissues stained with phthaleins. The results of the various experiments were in complete accord in showing that there exists a group of tissues—matrix tissues—which become more alkaline when the blood is alkalinized; and a second group of tissues, parenchymal in constitution, which are to all appearance unaffected even when alkalinization is pushed to the extreme. Tests were now begun upon the effects of acid.

Effects of Acid on the Coloration with Phenol Red.

There are few tissues staining with phenol red of which any color alteration can be expected on the injection of acid; for only a few have internal conditions bringing them within the range of this indicator, the reaction of the others appearing to lie beyond it, on the acid side (20). The connective tissue and tendons stain yellow-pink and pinky yellow respectively; and these colors might conceivably be altered to a frank orange-yellow when acid is administered. This has proved to be the case.

When $N/6.35$ HCl was gradually run into a jugular vein of the stained rat the body surface, which had been *jasper red*, became *coral red*, then *carnelian red* and eventually *rufous*. Meantime the connective tissue turned from salmon to orange-yellow, and the tendon, which had not been so pink to begin with, took on the same hue. The blood and lymph became somewhat less rosy than usual. The other tissues, being ordinarily orange-yellow, did not alter in hue. There was some diuresis, of acid urine stained with phthalein, but the changes noted in the color of the tissues, cannot be ascribed to an unusually great elimination of dye: for the application of alkali to specimens from the control and experimental animal rendered them equally and deeply rose-purple.

Effects of Acid on the Coloration with Brom Cresol Purple.

An extensive series of observations were carried out with brom cresol purple. The blood and lymph did not alter notably in hue as acidosis developed, yet there were great changes in the surface color, the hue turning from the *dull violet-blue* of Ridgway to *grayish violet-blue*, or, if the animal was more deeply stained to begin with, from *deep soft blue-violet* to *grayish blue-violet*. The alterations were mainly the outcome, as further findings showed, of changes in the hue of the connective tissue.

to the blood produced in this way under the circumstances of the experiments was not of itself sufficient to explain the issue of them. In a later paper some figures will be given on the anemia produced in rabbits by the intravenous injection of the maximum quantity of hydrochloric acid compatible with life. The blood loss is surprisingly slight.

How now are the changes in the relative reaction of the tissues to be accounted for? By changes in the ability of the blood to carry oxygen and carbon dioxide? This factor may have importance in the case of the animals given acid, owing to the effect of the latter to lessen the affinity of hemoglobin for oxygen, and thus to reduce the effective concentration of the gas. Asphyxia of the tissues is a well known cause of acid formation within them; and toward the end of some of our experiments the animals manifested air hunger. Acid lessens as well the ability of the blood to transport carbon dioxide; and the presence of this gas in the tissues of the stained, acidotic animal can be sufficiently shown by laying them open, when they rapidly become more alkaline, as evidenced by a change in hue. But this happens in the case of the similarly ventilated tissues of controls as well (22). When one alters the reaction of the blood in the direction of acidity one alters also the gradient responsible normally not alone for the passage of carbon dioxide from the tissues into the blood but for that of the acid products of metabolism generally. Here is yet another possible reason for the acidosis. This may have been due in no inconsiderable part to a damming back of substances which would normally escape to the circulation and be excreted, instead of to an extravascular penetration of hydrogen ions introduced with the injection fluid.

May one suppose that under the circumstances of blood alkalosis the gradient just referred to becomes so steep that the tissues are stripped, so to speak, of carbon dioxide and other acid products as soon as these are formed, with result that the local reaction becomes more alkaline than the normal? Such an occurrence is conceivable, since stained normal tissues ventilated by exposure to air soon become as alkaline as they ever appear to be after an injection of alkali.

The tissues which alter in reaction when alkali or acid is run into the organism,—connective tissue, cartilage, and tendon,—are those

continuously kept up not even a change in the surface color took place. By hurrying the injection a surface change from *jasper red* to a hue between *carnelian red* and *vinaceous-rufous* could be brought about, and under such circumstances alterations were to be noted in the color of the connective tissue, cartilage, and tendons, resembling those when hydrochloric acid had been used. But as soon as the injection was intermitted the surface color began to return toward the normal, as doubtless did the hue of the tissues mentioned, though observations were not made upon the point.

DISCUSSION.

The foregoing experiments make clear the fact that when acidosis or alkalosis is induced by the gradual injection of hydrochloric acid or of sodium carbonate into the blood stream changes take place in the reaction of some of the tissues. We have not sought to determine how great an amount of acid or alkali is required to cause these changes, nor have we made detailed observations upon the influence of the rate of injection. In most instances enough of the solution to cause death was eventually run in, but the examination was made while the animal was still in good condition save in the case of those tissues which the preliminary work had shown to be resistant to change. The solution could be allowed to enter rapidly at first, because of the buffering action of the blood, but later only drop by drop, so timed that each drop took some seconds or minutes to flow in. At first, too, because of the buffering action just mentioned, no change in the color of the tissues occurred. A few drops of the solution given late in the injection period often had more effect than did as many cc. earlier.

The question may be asked whether the changes were not the result of gross injury to the walls of the blood vessels with effusion of the acid or alkali. This possibility can be ruled out at once. The animal organism will tolerate but slight alterations in the reaction of the blood; and the color phenomena elicited by the injection of weak solutions ($N/6.35$) were the same as with strong ones ($N/2$ $N/1$). Further, the acid and alkali were of a kind to yield innocuous salts. It is true that even $N/6.35$ HCl acts to transform into acid hematin the hemoglobin coming directly into contact with it. But the damage

end in its effects upon the tissue reaction as if the disturbance had been a general one. That in pronounced cases an alteration takes place in the reaction of the matrix tissues can scarcely be doubted.

There is much on record to show that the introduction of acid into the body leads to a withdrawal of base from the tissues; and the quantity lost may well alter the composition of these latter. One wonders whether the matrix tissues, which are so readily permeable from the blood, may not serve as a second line of buffers, acting as interstitial tissues, tissues that fill up cracks, in a physiological as well as an anatomical sense. It would be in keeping with what is termed the body economy if a material like cartilage should prove useful in other ways than by keeping the ears at an angle from the head and the joints of the limbs well rounded.

Somewhere in the literature surely, there must be indirect observations upon the participation of the tissues in the buffering activities so beautifully illustrated by the blood; for the extent of the tissue participation can be come at, since the total blood value as a buffer is calculable and the changes incident to excretion can be controlled. The only observations we have been able to find, however, are those of Atzler and Lehmann (25) who perfused the isolated hind legs of dogs with acid and alkaline solutions over long periods of time and came to the conclusion that the greater the initial degree of acidity or alkalinity the greater was the change toward neutrality undergone by the perfusate.

Our observations by the indicator method upon the changes occurring in the tissues when acid or alkali has been run into the blood stream are not the first upon this theme. Henning (26) injected HCl or NaOH into mice and sought to determine the reaction of the organs after the method of Gräff (27) by dipping pea-sized pieces of them into drops of indicator solution placed on a glass slide. The animals had been decapitated, or had died as direct result of the injection; and the specimens stained after death contained the blood into which acid or alkali had been run. Under these conditions Henning noted that when a great deal of acid or alkali had been placed in circulation practically all of the tissue pieces except those from the brain appeared to contain H or OH ions in abnormal quantity.

characterized by the presence of matrix substance in large quantity, a matrix which stains deeply with the phthaleins. As pointed out in previous papers these tissues appear to be largely passive in behavior. They control their own conditions only where the influence of the separated cells is immediately effective; and when stained with phthaleins and immersed in weak buffer solutions they behave almost like so much indicator paper (23). Their metabolic rate is very low as compared with that of the parenchymatous organs. One may ask whether the intercellular substances which constitute so large a part of their bulk are alive at all in any proper sense of the word. It is easy to suppose that the changes in the reaction of these tissues are the direct result of an exchange of ions between them and the altered blood, without invoking other causes. But the precise interpretation of our findings cannot now be attempted for lack of data.

To give reasons for the failure of the parenchymatous organs to undergo changes in reaction is another, and more difficult matter. True, the cells of these organs may be thought of as making their own conditions (24), one of these being under ordinary circumstances,—or so it would appear,—a not inconsiderable acidity. But there is no evident increase in this local acidity in animals rendered acidotic, despite the great metabolic activity of the cells; and no diminution in it even when alkalosis is pushed to the extreme. It is possible, not to say certain, that the abilities of cells to maintain a normal state of affairs within their cytoplasm are often greater than those for survival of the body as a whole. Yet quite possibly too the phthalein of our experiments may in the parenchymatous organs have become so associated with some of the cell components that it no longer served as an indicator. All of the observations made in this laboratory prior to the ones here reported are against this explanation, however.

The changes toward acidosis or alkalosis sustained by the organism under the conditions of nature differ from those we have reported in that they are not ordinarily caused by a direct introduction into the blood of H or OH ions from without, though often referable to an indirect passage of them into the circulation by way of the gut. A large proportion of clinical instances of alkalosis or acidosis derive from some upset here or there amongst the organs. But except in the immediate region of the upset the net result must be the same in the

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Some of the rats of the present work withstood so well the introduction of N/6.35 HCl and the resulting marked changes in tissue reaction that we have been led to make observations upon the course of tissue acidosis as thus induced. The work constitutes an elaboration in certain directions of that here reported. It will be the subject of a succeeding report.

SUMMARY.

The introduction into the blood stream of dilute hydrochloric acid or sodium carbonate in quantities not too great to be compatible with life results in marked alterations in the color of certain of the matrix tissues stainable *in vivo* with phthalein indicators. Connective tissue in its various forms, and tendon and cartilage all become relatively more acid or alkaline than the normal. The hue of the kidney cortex also changes, as might be expected from its functions. The pancreas and lymph nodes, on the other hand, appear unaffected even when the injection is pushed to the extreme; and the slight to negligible alterations in the hue of the liver may be due to changes in the color of the associated secretion. The matrix tissues just mentioned behave as if unable to maintain a reaction of their own; whereas the elements of the parenchymal organs would seem to make their own conditions even when so much acid or alkali has been injected as will lead to death of the animal.

Injections of lactic acid are well tolerated and it is difficult to bring about alterations in the color of the phthalein-stained matrix tissues by means of them. Salt solution, and sugar in large amount cause no changes in color such as would indicate changes in reaction.

The results with the various phthalein indicators are in close accord, attesting that the information these give under vital conditions is reliable.

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deeply with acid dyes. The depth of staining depends to some degree, however, upon the length of exposure. The red-staining material may occupy not more than half, or even less, of the nuclear space or it may occupy almost the entire nucleus. In any case it is sharply limited from the surrounding nuclear material by a clear unstained halo. The material itself is usually granular, never hyaline or dense; it appears as though composed of a multitude of very fine granules compressed into a ball or mass.

Nuclear changes which cannot at present be differentiated from those occurring in the lesions of spontaneous and experimental herpes simplex also occur in the skin lesions of herpes zoster, in the skin lesions of varicella, and in the lesions experimentally produced in rabbits by the Virus III of Rivers and Tillett (2). Nuclear changes resembling to some extent those occurring in herpes simplex are also found in a variety of conditions, especially diseases of animals, such as, epidemic encephalomyelitis of horses, or Borna's disease, fowl-pox, certain diseases of fishes, etc.

Except for a few reported instances, nuclear changes like those occurring in herpes simplex have so far been found only in conditions in which the presence of a filterable virus has been demonstrated or in which the association of a virus of this group is very probable. The experimental production of these lesions except by the injection of filterable viruses has, in our hands, been unsuccessful. It is true that Luger and Lauda (4) have mentioned the occurrence of similar structures in a case of salvarsan dermatitis. But even though these lesions should be present in isolated instances of this kind, it would be necessary to demonstrate the absence of a filterable virus in the given instance before the present conception of the direct relationship between these nuclear changes and filterable viruses would become untenable.

Lipschütz (3) has considered that these nuclear changes represent "nuclear inclusion bodies" in the sense of Prowazek. He has collected all the conditions in which nuclear inclusions occur, and also the conditions in which unusual bodies or structures are found in the cytoplasm of the cells, into a great group of virus diseases, the causative agent of which he classes with the *Chlamydozoa-strongyloplasma*. Lipschütz has maintained that the bodies or structures seen within the nucleus represent a specific reaction of the cells to a living virus.

A FILTERABLE VIRUS PRESENT IN THE SUBMAXILLARY GLANDS OF GUINEA PIGS.

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PLATE 33.

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During recent years much attention has been paid to peculiar alterations in the morphology of certain cells in lesions associated with the presence of filterable viruses. These changes are especially constant and well marked in the lesions of spontaneous and experimental herpes simplex. Indeed, they occur so constantly in this condition that Goodpasture and Teague (1) have used the presence of these cells as a guide in tracing the passage of the virus through the tissues of the infected animal. The uniformity of the cellular changes in herpes simplex and the constancy with which they occur renders this the most satisfactory of the virus diseases to be used as an example in discussing analogous and related conditions. Cells derived from the endothelium, epithelium, or mesenchyme may show these changes and although there may be variations in the appearance of the alterations in the different cells, on the whole they are very similar, whether the cells involved be connective tissue cells, epithelial cells of the cornea, or large ganglion cells of the central nervous system.

The characteristic features of these changes, as seen in tissues stained with eosin and methylene blue, are the following. The nucleus takes on a vesicular character and the limiting membrane is deeply stained with the basic dyes. Usually the inner surface of the membrane is irregular, as though the basic staining material were collected in granules or small clumps. A few of these granules are much larger than the others and these are considered to represent the nucleoli of the unaltered nucleus. Within the nucleus, usually at the center, is a round or oval body which stains either faintly or, more often,

In a few isolated instances, pathologists have reported finding peculiar nuclear changes in human pathological material. VonGlabn and Pappenheimer (5) have recently collected the reports of sixteen human cases, and have added a case of their own, in which, at autopsy, in various viscera, cells with striking nuclear abnormalities were present. While the descriptions of these nuclear changes in the individual cases differ to some extent, in most instances they are sufficiently alike to justify the conclusion that the various writers were dealing with identical or closely related conditions. All of the cases reported, except that of VonGlabn and Pappenheimer, were in infants under 1 year, five of these were still-born. Five of them probably suffered from congenital syphilis, in one nephritis was present, in one pneumonia, one had "green stools," edema of feet, and bronchitis, another one hydrocephalus and focal interstitial nephritis. The case of VonGlabn and Pappenheimer was that of a male aged 36 years, who suffered from an abscess of the liver. Certain cells in the intestines, liver, and lungs were found to be of very large size, measuring at times 25 micra in diameter. Some of these were multinucleated. Within the nuclei of these cells were large acidophilic masses. In the lung these large cells were usually in continuity with the epithelium, but cells of granulation tissue and of the blood vessels also contained the nuclear inclusion masses.

In addition to these cases collected by VonGlabn and Pappenheimer, L. Jackson (6) has reported finding an ameba-like organism in the kidneys of a child. This child, 15 months old, suffered from diphtheria and died suddenly. At autopsy lesions of bronchopneumonia were found. In the kidney tubules were large cells which the writer interpreted as amebæ. It seems from the description that these may have been structures similar to those present in the cases collected by VonGlabn and Pappenheimer. Indeed, Jackson stated that the structures were similar to those described by Ribbert (7) and by Smith and Weidman (8), whose cases were included by VonGlabn and Pappenheimer in their series.

In most of the earlier human cases the nuclear inclusions were thought to be due to the presence of parasites, amebæ, or sporozoa, in the later cases they were described as nuclear degenerations. One of the cases in infants was reported by Goodpasture and Talbot (9) in 1921. These writers drew attention to the similarity between the nuclear changes in this case and the nuclear changes in the epithelial cells in varicella which were first described in 1906 by Tyzzer (10). They also called attention to peculiar cellular changes present in the epithelial cells of the salivary glands of guinea pigs which were first described by L. Jackson in 1920. Goodpasture and Talbot studied these cells in guinea pigs and concluded that the condition was an example of cellular transformation similar to that occurring in the lesions of infancy. They thought that there was no evidence that these cellular and nuclear changes were due to some intracellular infection. VonGlabn and Pappenheimer were the first to fully identify the large cells occurring in human cases with the abnormal cells met with in herpes simplex and related conditions. They regarded the intranuclear masses seen in their own case as nuclear inclusions, "identical in their morphology and staining reactions with the

The bodies are not considered to be masses of parasites but are held to represent reaction products, associated with which is the virus.

This conception of Lipschütz has not been universally accepted, however. Luger and Lauda (4) who have devoted much study to the nature of these nuclear changes, maintain that they do not represent "inclusion bodies" in the sense of Prowazek, but that they are the result of a non-specific type of nuclear degeneration, which these authors call "oxychromatic degeneration." They refer to the observations of Heidenhain, who described two varieties of nuclear, chromatic substance, basic chromatin and oxychromatin. During nuclear degeneration occurring under certain conditions, the oxychromatin tends to collect in the center of the nucleus and the basic chromatin at the periphery. According to these writers the typical nuclear changes seen in the lesions of herpes simplex represent the final stages in this separation of oxy- and basic chromatin.

At the present time the evidence in favor of either of these views is not convincing. Our own observations suggest that the inclusion bodies are produced by the accumulation of a finely granular material which at first is scattered throughout the nucleus. This material which, in its scattered form, is very faintly acidophilic, takes on a deeper and deeper red stain as it accumulates into a mass, until the typical nuclear changes become manifest. The earlier stages of this process, however, are difficult to detect and usually only the fully developed nuclear "inclusion body" is seen. Until further knowledge concerning the chemical nature of these structures is obtained, it is not important, at least in the present connection, to decide whether we shall speak of "nuclear inclusions" or of "oxychromatic degeneration" of the nucleus. It is very important, however, to know whether these changes represent characteristic lesions due to the action of filterable viruses or whether they represent a form of degeneration which occurs under a great variety of conditions producing a non-specific injury to the cell. The burden of evidence at present points to the former concept, for it would be very surprising, in view of the careful study which has been made of cellular changes under various pathological conditions, that this striking nuclear alteration should have been frequently overlooked.

of these structures can be seen within a single duct; in other ducts not more than one or two of these cells are present. In the sections from certain glands only one or two groups of ducts containing the altered cells are seen, in other glands large numbers of ducts are involved. About the ducts containing these cells there is usually a cellular reaction and this is often of assistance in locating the areas in which the altered cells are present. The tissue reaction about the involved ducts consists of mononuclear cells, lymphocytes, and large cells with vesicular nuclei (Fig. 3). Polymorphonuclear leucocytes are rarely seen and never predominate. The cytoplasm of the involved cells usually takes on a light blue color which, however, is considerably more intense than that of the cytoplasm of the unaltered cells. The nuclear membrane is somewhat irregular, and stained deeply with the basic dye. At or near the center of the nucleus, and separated from the membrane by a clear unstained area or halo, is a mass which is stained red. The color of this mass is usually definitely deeper than that of the typical herpes simplex inclusion bodies. This mass may occupy not more than one-fourth the nuclear space but it not infrequently fills almost the entire space, leaving a very narrow halo between it and the membrane. Within this clear space there are seen two, three, or even more, irregular masses, 0.5 to 2 micra in diameter. These masses stain deeply with the basic dye. They may lie approximated to the inner surface of the membrane, or upon the acidophilic mass, or free in the space. Upon careful focussing in almost all instances radiating irregular lines passing from the mass to the inner surface of the membrane can be observed. These bands also stain with the basic stain. Occasionally a very faint network of strands between the various radiating bands is apparent. The central mass is usually oval or round, the margin is slightly irregular, and the mass appears finely granular. At times some of the granules appear larger and somewhat refractile. The presence of these granules may have been responsible for the interpretation of these structures as protozoa and for the descriptions of an organized structure. It is evident from this discussion that in their main features the cells resemble those occurring in the lesions of herpes simplex. On account of their much greater size and the darker color of the inclusion bodies the resemblance is, at first, not evident.

Sections have been made from the submaxillary glands of a number

bodies seen by previous observers in the viscera of infants, and by Lipschütz and others in tissues of spontaneous and experimental herpes, and in various neural and visceral lesions produced by the herpetic and related viruses." These writers were unable to carry on any experimental study, but offer the suggestion that the lesions in this case may have been related to the presence of an unknown virus.

Cellular Changes in the Submaxillary Glands of Guinea Pigs.

Unusual changes in the submaxillary glands of guinea pigs were first described by L. Jackson (11) under the title, "An intracellular protozoan parasite of the ducts of the salivary glands of the guinea pig." These structures were found in 26 of 48 pigs examined. She interpreted them as protozoa, probably coccidia. We have had no difficulty in confirming the observation of Jackson that in the ducts of the submaxillary glands of guinea pigs there are found unusual and striking structures which on first observation suggest a parasitic origin. However, we have been unable to differentiate the different stages of development resembling those of protozoa which Jackson described. The structures we have observed, however, conform in all particulars with those shown in the illustrations in Jackson's paper and there can be no doubt that we are dealing with the same abnormalities. We have examined the submaxillary glands of 75 guinea pigs over 6 months of age. Sections from these glands have been stained in eosin and methylene blue, and in 63 of the glands, or 84 per cent, these unusual structures have been found in larger or smaller numbers. From our own study we have identified them as swollen epithelial cells (Fig. 1). The nucleus of each of these cells contains a mass of granular material which is definitely acidophilic. The altered cells are found chiefly in the ducts of the serous portion of the gland, though, in a few instances, they have been seen in the mucous portion. The cells lie either on the basal membrane contiguous to the unaltered epithelial cells or within the lumen of the duct, evidently having pushed forward during the process of hypertrophy. The altered cells occasionally are not more than twice the usual size (Fig. 2) but in most instances they are much larger than this, up to 40 micra in diameter. The large size and red staining of the nuclear inclusions render them easily visible under the low power of the microscope. In ducts cut obliquely or longitudinally, not infrequently six or eight, or even more,

somewhat swollen but they did not equal in size the very large cells in the glands of full grown guinea pigs. Most of the transmission experiments, however, have been made by injecting the material in other locations in young pigs where these cells do not occur, such as the testicle, brain, lung, and tongue. The most striking results were obtained when the injections were made into the brain and in most of the experiments this has been the site of inoculation.

Minor modifications of the technique of the brain injections were made in certain experiments and the results were not always identical but the following description is typical of this experimental method and of the results obtained. A full grown guinea pig is killed and the submaxillary glands removed under sterile precautions. A small piece is removed from each gland and placed in Zenker's solution, to be later examined in order to make certain that typical lesions are present. Aerobic and anaerobic cultures of the submaxillary gland are made at this time to exclude the possibility of bacterial infection. The remainder of the glands is cut into small pieces with scissors and ground in a mortar in about 2 cc. of Locke's solution. This is then centrifuged at low speed for a few minutes, to remove the large particles, and the supernatant fluid is used for injection. 0.1 cc. of this suspension is then injected directly into the brain¹ of a guinea pig less than 1 month old. 24 hours following this injection the temperature of the injected pig is usually not elevated and the animal appears normal. After 48 hours, however, the temperature frequently becomes elevated, 105–106°, but without the animal showing any marked symptoms. On the 3rd day the guinea pig appears sick, the hair is raised, the animal fails to move about in the cage, and the temperature continues elevated. On the 4th day, the symptoms have become more marked. The animal now begins to show signs of heightened nervous irritability as indicated by tremors and slight convulsive movements. On the 5th day it is usually very ill, has irregular jerking movements, is unable to rise when placed on its side, and death ensues. When the brain is removed no gross abnormalities beyond congestion are seen. Cultures are made to rule out the possibility of bacterial infection, and the brain is placed in Zenker's solution.

¹ Guinea pigs were anesthetized with ether before injection.

of animals of other species to determine whether identical or analogous structures are also present in them. The glands of eight full grown rabbits, three rats, a mouse, a dog, and a cat, have been studied but no structures resembling the large cells seen in guinea pigs have been found. Although these peculiar cells were present in the submaxillary glands of 84 per cent of the guinea pigs over 6 months old, in very young pigs they were present only occasionally. Of 43 young guinea pigs, most of them less than 1 month old, the large cells were found in the submaxillary glands in only three instances and then they were few in number and confined to one or two groups of ducts. It seems, therefore, that if these lesions are due to an infection with a virus, the infection usually occurs only after the first few weeks of life but finally almost all guinea pigs become infected.

EXPERIMENTAL.

Transmission of Infection to Young Guinea Pigs.—An attempt was first made to demonstrate whether or not an infectious process is responsible for these lesions. The most obvious mode of procedure would be the inoculation of material from glands in which the lesions were presumably present into other glands which were not already the seat of the lesion. Since, however, the lesions are found in almost all older guinea pigs it would be necessary to inject the material into the glands of very young guinea pigs free from infection. Injections into the submaxillary glands of very young guinea pigs, however, are not without difficulties, and moreover, there is always the chance that even the young guinea pigs are already infected. Nevertheless, a number of experiments have been made in which material from the glands of old guinea pigs was injected into the glands of young ones. The pigs were killed at varying periods, from 2 to 12 days, following the injection and sections were made from the submaxillary glands. In nine out of eleven injected glands a marked mononuclear reaction involving the interstitial tissue was present. In certain areas cells were found which showed nuclear changes, with acidophilic inclusions, exactly resembling those seen in the lesions of herpes simplex. Occasionally similar nuclear changes were seen in duct cells of normal size. In a very few instances the duct cells showing these changes were

were either in cells of the interstitial tissue, or in cells of the tubules, or in both.

Injections of an emulsion of the submaxillary glands of full grown guinea pigs were also made into the tongues of young guinea pigs. These animals developed no symptoms. They were killed and the tongues were removed on the 3rd, 5th, and 9th days following the injection. Microscopic study of these tongues showed a localized cellular reaction in the stroma, and cells containing typical nuclear inclusion bodies were present. A tongue removed on the 9th day after inoculation showed the most marked cellular infiltration. In no instance was the epithelium of the tongue involved.

Inoculations were also made into the lungs of three guinea pigs. One of these animals showed a rise of temperature on the 7th day, and was killed. The lung showed no gross changes but on microscopic examination a circumscribed mononuclear cellular infiltration was found. In a few of the alveoli in the involved area, large mononuclear cells containing typical nuclear inclusion bodies were present. Two other guinea pigs inoculated in the lungs were killed on the 11th and 22nd days after injection. Although a mononuclear cell infiltration was present in the lungs, no cells with nuclear inclusion bodies were found. The injection of emulsions of submaxillary glands of full grown guinea pigs into young guinea pigs therefore has quite regularly resulted in the production of a subacute inflammatory reaction and the appearance of cells containing nuclear inclusion bodies morphologically identical with those seen in herpes simplex and allied conditions.

Control Experiments.—These experiments suggest that the submaxillary glands of full grown guinea pigs contain a virus which is responsible for the reaction in the young guinea pigs, and it seems probable that the large cells and the reaction seen in the older pigs is a manifestation of natural infection with this virus. This view is supported by the following observations. Four young guinea pigs were inoculated in the brain with an emulsion of the submaxillary glands of other young guinea pigs, presumably tissue containing no large cells with nuclear inclusions. Subsequent microscopic examinations showed that there were actually no lesions in these glands. The results of these inoculations were entirely negative. Nevertheless, it seemed possible that the reaction observed in the tissues of young guinea pigs

Later when microscopic sections are examined there is found well marked exudate over the surface of the entire brain, including the cerebellum (Fig. 4). The exudate consists chiefly of mononuclear cells, lymphocytes, and large cells with vesicular nuclei. There is considerable edema and the blood vessels of the membranes are distended and filled with blood. In contrast with the lesions resulting from intracerebral infection of the brain with herpes simplex virus, very slight if any changes can be detected in the brain itself, the blood vessels of which appear normal. The most striking feature is the presence in the meningeal exudate of large numbers of cells, each of which contains an acidophilic mass within the nucleus. These cells resemble in all particulars the cells containing nuclear inclusion bodies which occur in herpes simplex and related conditions (Fig. 5). The number of abnormal cells present in the guinea pig lesion is much greater than the number usually present in the lesions following injection of herpes simplex virus or Virus III of Rivers. In no case have very large cells like those found in the submaxillary gland been found. 54 young guinea pigs have received intracerebral inoculations of an emulsion of the submaxillary gland of full grown guinea pigs. Most of these animals showed symptoms similar to those described, though in some the symptoms were delayed. The animals died or were killed at various times from the 2nd to the 12th day following the injection. In 48 of the guinea pigs, or 89 per cent of those injected, lesions as described above were found.

Emulsions of the submaxillary gland of full grown guinea pigs have also been injected into the testicles of sixteen young guinea pigs. In all cases a histologic examination of the submaxillary gland was made to make certain that the specific lesion was present in the material used for injection. 0.1 cc. of the emulsion was usually injected into each testicle. The inoculated guinea pigs showed some elevation of temperature for several days, but they developed no other symptoms. The animals were killed at various intervals from 4 to 10 days following the injection, and both testicles were removed. Histologic examination was later made of one testicle from each animal and in almost all cases some degree of cellular infiltration was found. In twelve instances, or in 75 per cent of the animals inoculated, cells containing typical nuclear inclusion bodies were present. These

the seat of inoculation, inoculating first in the brain and then in the testicle or *vice versa*. A large number of these experiments have been carried out and in seven instances has it been possible to obtain a positive result on the second transfer. In three of these experiments the transfer was made from brain to testicle, in three others from testicle to brain, and in one from testicle to testicle. In one series it has been possible to reproduce the lesions through a series of three transfers (Series T). In this instance the transfer was made through testicle, brain, testicle. The following are abstracts of the protocols of the experiments in which positive results were obtained.

Series M—Brain to Testicle.—March 1, 1926, Guinea Pig 1 received an injection into the brain of 0.1 cc. of an emulsion of the submaxillary glands of two full grown guinea pigs. In this animal the temperature was elevated from the 3rd to 7th days following the injection, reaching 105.8°. The guinea pig showed marked symptoms, became prostrated, and was killed on the 8th day. Sections from the brain of this animal showed a marked meningeal exudate, and typical nuclear inclusions were found in many of the cells. The remainder of the brain was emulsified and 0.1 cc. of the emulsion was injected into each of the testicles of Guinea Pig 2. In this animal the temperature was elevated to 104.4–105° from the 3rd to the 11th days. It was killed on the 11th day. Sections from the left testicle showed a mononuclear infiltration and a few cells contained typical nuclear inclusions.

Series N—Brain to Testicle.—March 26, 1926, Guinea Pig 3 received an intracerebral injection of 0.1 cc. of an emulsion made from the submaxillary glands of two full grown guinea pigs. The animal became very sick on the 3rd day and was killed. Sections from the brain showed a marked meningeal infiltration with numerous cells showing typical nuclear inclusions. The remainder of the brain was emulsified and 0.1 cc. of the emulsion was inoculated into each of the testicles of Guinea Pig 4. The animal showed an elevation of temperature from the 7th to the 9th days, ranging from 104.4–105.2° and was killed on the 9th day. Sections from the testicle showed a slight cellular reaction and in a few cells typical nuclear inclusions were found.

Series P—Brain to Testicle.—March 18, 1926, Guinea Pig 5 received an intracerebral injection of 0.1 cc. of an emulsion of the submaxillary glands of two full grown guinea pigs. The animal showed an elevation of temperature and marked symptoms, and died on the 4th day. An emulsion made from the brain of this animal was injected into both testicles of two young guinea pigs, Nos. 6 and 7. On the 7th day Guinea Pig 7 showed a temperature of 105.4° and was killed. In sections of the testicles, however, no lesions were found. Guinea Pig 6 had a temperature ranging from 104.9–105.6° on the 7th to 9th days. The animal was killed on the 9th day and in sections of the testicle a circumscribed mononuclear

following the injections of the glands of old guinea pigs was of a non-specific nature, and that any glands of external secretion might contain irritating substances which on injection into the brains of young guinea pigs might give rise to an inflammatory reaction, and that under these conditions cells containing nuclear inclusions might appear. Consequently, two young guinea pigs were injected with emulsions made from the pancreas of an old guinea pig and seven young guinea pigs were inoculated with emulsions made from the submaxillary glands of full grown rabbits. No symptoms resulted from any of these inoculations and the microscopic examination of the brains failed to reveal any lesions. Moreover, nine young rabbits, inoculated intracerebrally with an emulsion made from the submaxillary glands of full grown rabbits, remained well and no lesions were found in the brain. The results so far reported, therefore, indicate that an infectious agent is probably responsible for the lesions found in the submaxillary glands of full grown guinea pigs and that the lesions in young guinea pigs resulting from the inoculation of the emulsion of submaxillary glands of full grown guinea pigs are due to this infectious agent.

Transmission in Series.—This conclusion would only be justifiable, however, if it were possible to reproduce the lesion after passage through a series of animals. But in spite of the fact that young guinea pigs which have received intracerebral inoculations of emulsion of submaxillary glands of old guinea pigs almost invariably show severe symptoms with marked cerebral lesions, the inoculation of emulsions of the brains of these experimentally infected animals into other young guinea pigs has invariably failed to cause symptoms or to give rise to lesions. But even though the second animal has shown no lesions or symptoms the inoculations have been continued from animal to animal in series through as many as six guinea pigs with the hope that in this way the virus might gradually acquire greater virulence. But the results have all been negative. Attempts were also made to transmit the virus by inoculating from testicle to testicle or from salivary gland to salivary gland. Except in one instance, to be mentioned later under Series C, when a testicle to testicle inoculation gave a mildly successful result, these experiments were also unsuccessful. Recourse was then had to the expedient of varying

showed a slight cellular infiltration and edema and in the tubules a small number of cells with nuclear inclusions were found.

It will be seen from these protocols that in seven instances it has been possible to produce lesions in two animals in series and in one other instance in three animals in series. Many variations in the technique have been made in the hope of transmitting the virus indefinitely. Transfers were made at various periods following the infection, even as early as the 2nd day. In other experiments, instead of employing the entire brain tissue for the emulsion, only scrapings from the surface of the brain were used, since the lesions containing the cells with nuclear inclusions are found only in the meningeal exudate. In other experiments it was thought possible that some stimulating or accessory substance present in the submaxillary gland might be necessary for infection and that when transfers were made from brain to brain this factor would of course be lacking. Consequently, emulsions of submaxillary glands of very young pigs were added to the brain emulsions of infected guinea pigs which were used for transfer. The results with none of these methods, however, proved successful.

Although it was not possible to transmit the virus indefinitely, the results obtained offer considerable evidence that in this condition we are dealing with an agent which reproduces itself, and, therefore, presumably is a living virus. There has been no indication so far obtained that the virus on passage tends to become more virulent. Indeed, the opposite effect has been observed. In all cases when any effect has resulted from the second transfer the lesions have been less well marked than those following the first transfer. Although the virus may be preserved, at least for short periods, in glycerol, no evidence was obtained that any increase in virulence or infectivity occurs during this time.

Infectiousness for Other Species of Animals.—Attempts have been made to reproduce the lesions in other species by the inoculation of an emulsion of the submaxillary glands of full grown guinea pigs into the brains of nine young rabbits, five young rats, and two young kittens. These animals all remained well and sections of the brains of these animals, which were killed at varying intervals, showed no lesions.

reaction was seen and in the cells of the tubules in this area there was found a small number of cells, in the nuclei of which were typical acidophilic masses.

Series C—Testicle-Brain: Testicle-Testicle.—January 5, 1926, Guinea Pig 8 received an injection into the left testicle of 0.1 cc. of an emulsion made from the submaxillary gland of an adult guinea pig. This animal had an elevated temperature from 104.8–105.7° on the 6th, 7th, and 8th days, and it was killed on the 8th. Sections made from the testicle showed a marked infiltration with mononuclear cells, and in a small number of the tubule cells, typical nuclear inclusions were found. The remaining portion of the testicle was emulsified in Locke's solution and 0.1 cc. was injected into the brain of Guinea Pig 9 and into the left testicle of Guinea Pig 10. Guinea Pig 9 showed an elevation of temperature on the 5th to the 9th days, ranging from 104.7–105.8°. On the 9th day this animal was killed and sections made from the brain showed a circumscribed meningitic exudate in which were numerous cells showing typical changes with nuclear inclusion bodies. The testicle of Guinea Pig 10 was removed on the 9th day. Sections from this showed a very slight interstitial reaction, a few of the cells of which showed typical nuclear inclusions.

Series T—Testicle-Brain-Testicle.—April 15, 1926, Guinea Pig 11 received an inoculation into the left testicle of an emulsion made from the submaxillary glands of two full grown guinea pigs. The temperature was elevated on the 6th and 7th days, rising to 106° on the 8th day. On this day the animal was killed, the testicle removed, and placed in 50 per cent glycerol. On the following day the testicle was washed free of glycerol and emulsified in Locke's solution. 0.1 cc. of this emulsion was injected into the brain of each of two guinea pigs, Nos. 12 and 13. Beginning with the 3rd day following the injections, both animals showed an elevation of temperature, ranging between 104° and 106°. On the 7th day Guinea Pig 12 was killed. A small piece of the brain was retained for microscopic study and the remainder was placed in 50 per cent glycerol. On the 9th day following the injection, Guinea Pig 13 was killed, the brain removed, a small piece placed in Zenker's fluid for microscopic study, and the remainder placed in 50 per cent glycerol. The examination of the sections of the brains of Guinea Pigs 12 and 13 revealed, in each case, a localized meningeal reaction, and a moderate number of the cells showed characteristic changes with the nuclei containing acidophilic masses.

The brains of Guinea Pigs 12 and 13 were preserved in glycerol for 25 and 27 days, respectively. An emulsion was made from this glycerolated material on May 28 and 0.3 cc. of this was inoculated into the brain of Guinea Pig 14 and 0.1 cc. into each testicle of Guinea Pig 15. Although Guinea Pig 14 had some fever there were no marked symptoms and it was killed on the 11th day following the injection. Sections made from the brain of this animal showed no lesions of any kind.

Guinea Pig 15 had moderate fever up to 105°. It was killed on the 11th day, and the testicles were removed. The left testicle was preserved for further inoculation and the right placed in Zenker's solution. Sections made from this testicle

injection and Guinea Pig 24 was found dead on the 10th day. Microscopic study of the brains of both of these animals showed a meningeal exudate containing cells which showed typical nuclear inclusion bodies.

In another experiment in which the submaxillary gland was exposed to 50 per cent glycerol for 7 days, a similar result to that described above was obtained. In another instance the submaxillary gland was exposed to 50 per cent glycerol for 28 days. The injection of this material into the brains of young guinea pigs failed to produce the characteristic cerebral lesions.

Filterability of the Virus.—On April 15 two full grown guinea pigs were killed and the submaxillary glands removed with sterile precautions. Sections prepared from small pieces of these glands were subsequently shown to contain the specific lesion in the ducts of the glands. Aerobic and anaerobic cultures of the glands made at this time remained sterile. The glands were ground thoroughly in a mortar and suspended in a total volume of 15 cc. of Locke's solution. The suspension was centrifuged at moderate speed for 15 minutes. Half of the supernatant fluid was then filtered through a new Berkefeld N filter. The material filtered rapidly. The filter was subsequently tested and found to be impermeable to *B. coli*. 0.1 cc. of the unfiltered suspension was inoculated intracerebrally into each of three guinea pigs, Nos. 26, 27, and 28. 0.15 cc. of the filtered material was inoculated intracerebrally into each of three guinea pigs, Nos. 29, 30, and 31. All the guinea pigs were less than 1 month old. The results following injection of the unfiltered material were as follows: On April 19, the 4th day following injection, Guinea Pig 26 was found dead, No. 27 was moribund, and No. 28 seemed sick and was killed and the brain removed for histologic examination. Microscopic study of the brain of No. 28 showed an intense meningitis containing numerous cells showing typical nuclear inclusion bodies.

The results following the injections of the filtered material were as follows: Guinea Pigs 29, 30, and 31 all showed on the 2nd day, a rise in temperature ranging from 105–105.6°. On the 4th day Guinea Pig 29 had a temperature of 105.2°. On the 5th day the temperature began to drop and on the 6th day it was subnormal and the animal was killed. The brain was removed and prepared for histologic examination. Guinea Pig 31 showed a rise in temperature, ranging from 104–105° from the 4th to the 8th days. The animal was killed on the 8th day and the brain was removed for histologic examination. The temperature of Guinea Pig 30 ran an irregular course. This animal was killed on the 12th day and the brain removed. A microscopic study of the brains of these three guinea pigs showed a moderate meningitis and in every instance cells containing typical nuclear inclusion bodies were found.

In a second filtration experiment a new Berkefeld N filter was used which was tested during the course of the filtration by the addition of 0.5 cc. of an 18 hour broth culture of *B. coli* to the suspension of submaxillary gland. Cultures of the filtrate remained sterile. The same result as the one described above, was obtained.

Properties of the Virus.

Thermolability of the Infectious Agent.—On February 3, a full grown guinea pig was killed and the submaxillary glands removed with sterile precautions. Histologic sections prepared from small pieces of these glands were subsequently shown to contain the specific lesions. Aerobic and anaerobic cultures of the glands made at this time remained sterile. The glands were ground thoroughly in a mortar and suspended in 5 cc. of Locke's solution. After centrifuging for a few minutes at low speed the suspension was divided into two parts; one half was heated at 54°C. for 1 hour and the other was stored on ice during this period. 0.1 cc. of the unheated suspension was then injected intracerebrally into Guinea Pigs 16, 17, and 18, and 0.1 cc. of the heated suspension was injected intracerebrally into each of the guinea pigs, Nos. 19, 20, and 21. All the pigs were less than 1 month old. One of the animals inoculated with the heated material, Guinea Pig 20, was found dead on the 2nd day after inoculation. The brain was removed and prepared for histologic examination. One of the animals which had been inoculated with the unheated material, Guinea Pig 18, was killed on the same day and sections were prepared from the brain for comparison with the sections from Guinea Pig 20.

On February 6, a marked contrast was noted between Guinea Pigs 16 and 17, inoculated with the unheated suspension, and Guinea Pigs 19 and 21, inoculated with the heated suspension. The former appeared unsteady on their feet and their hair was ruffled, whereas Guinea Pigs 19 and 21 appeared perfectly normal. All four guinea pigs were killed on the 3rd day following injection, and histologic sections were prepared from the brains. Microscopic examination of the brains of Guinea Pigs 16, 17, and 18 all showed a marked mononuclear exudate into the meninges in which numerous cells with characteristic nuclear inclusion bodies were found. On the other hand, sections from the brains of Guinea Pigs 19, 20, and 21 showed no meningeal exudate and no cells containing nuclear inclusions.

Resistance of the Virus to 50 Per Cent Glycerol.—On May 17 small pieces of the submaxillary gland of three full grown guinea pigs were placed in a small sterile bottle containing equal parts of glycerol and Locke's solution. The bottle was then stored on ice. A small piece of each gland was prepared for histologic examination and was subsequently shown to contain the specific lesion. The remainder of the glands was emulsified in the usual manner and 0.1 cc. was injected intracerebrally into each of two young guinea pigs, Nos. 22 and 23. Guinea Pig 22 was moribund on the 5th day following injection and was killed. Guinea Pig 23 was found dead on the 7th day. Microscopic study of the brains of Guinea Pigs 22 and 23 showed the usual brain lesion with typical nuclear inclusion bodies. On May 28, 11 days after placing the submaxillary glands in 50 per cent glycerol, the pieces of tissue were washed free of glycerol, ground in a mortar, and suspended in Locke's solution. After centrifuging a few minutes at low speed, 0.1 cc. was injected intracerebrally into each of the guinea pigs, Nos. 24 and 25, both less than 1 month old. Guinea Pig 25 was found dead on the 7th day following the

nuclei of the atypical cells in the lesions of herpes simplex. These cells are usually surrounded by a mononuclear cellular reaction. They were found in 84 per cent of the full grown guinea pigs examined but they were present in only three of forty-three young guinea pigs less than 1 month old. The resemblance of these cells, except as regards size, to the atypical cells present in lesions due to filterable viruses suggested that they also may be the result of an infection with a similar agent. That they are usually not present in guinea pigs less than 1 month old indicates that natural infection usually occurs after this period.

Experiments were therefore undertaken to determine whether or not an infective agent is concerned in this condition and if so to learn something of its nature. When an emulsion of the submaxillary glands of full grown guinea pigs is injected into the brains of young guinea pigs the animals have fever and exhibit symptoms of cerebral irritation. They usually die in 5 to 7 days and in sections of the brain a diffuse subacute meningitis is found. In the exudate there are large numbers of cells having all the characteristics of the abnormal cells of herpes simplex. Similar cells are present in the lesions resulting from the injection of the same emulsion into the testicle, lung, tongue, and submaxillary glands of young guinea pigs. In none of these lesions, however, are the cells greatly enlarged as they are in the lesions in old guinea pigs.

These results support the view that the lesion in the submaxillary gland of old guinea pigs is due to an infective agent. Attempts were therefore made to transmit this agent through a series of young guinea pigs. When the injections were all made into the same organ all the experiments but one gave negative results, but when the site of injection was changed at each transfer it was possible in a number of instances to reproduce the lesions through two animals in series and in one experiment through three animals in series. By modifying the technique, efforts were made to transmit the infection indefinitely but these attempts were unsuccessful. No explanation can be offered for this failure.

Studies made to determine some of the properties of the infective agent have shown that it is destroyed by heating at 54° for 1 hour, and that it is not injured by preservation in 50 per cent glycerol for as long as 11 days. After the material had remained in 50 per cent glycerol

Relation of the Infection of the Submaxillary Glands to Other Diseases of Guinea Pigs.

The only other disease affecting guinea pigs known to the writers which may possibly be related to the infectious process in the submaxillary glands is a condition described in 1911 by Römer (12). He observed sporadic cases of paralysis of the extremities in guinea pigs. By intracerebral inoculation of healthy guinea pigs with emulsions of the brains of the diseased animals he was able to transmit the infection without difficulty. After an incubation period of from 9 to 23 days the inoculated animals developed paralysis and after 2 to 10 days of severe illness, died. The brains of both the spontaneously and experimentally infected animals showed a marked infiltration of the meninges with an exudate containing many mononuclear cells and also many polymorphonuclear cells. No mention was made of the presence of cells containing nuclear inclusions.

The brain lesions in this condition resemble to some extent the lesions observed after intracerebral injection of the virus from the submaxillary glands. However, the ease with which the infection could be indefinitely transmitted and the long incubation period observed render it unlikely that the agents concerned in the two conditions are identical, though this possibility should be borne in mind.

SUMMARY AND CONCLUSIONS.

In the lesions of herpes simplex and similar conditions due to filterable viruses, cells are present which show characteristic alterations, particularly in the nucleus. The nucleus of these cells contains a mass which stains with acid dyes. Surrounding this mass is a clear space or halo, within which there are large granules staining with basic stains. These cells are little if at all enlarged.

In a few human cases, especially in infants, enlarged cells have been found which contain nuclei showing changes similar to those seen in the abnormal cells of herpes simplex.

In the ducts of the submaxillary glands of guinea pigs, Jackson observed structures which she considered to be protozoan parasites. Our own studies indicate, however, that these structures are greatly swollen epithelial cells with nuclei having the same characters as the

for 28 days, however, it was found to be no longer infective. The infective agent was not held back by a Berkefeld N filter which was impermeable to bacteria. It seems probable therefore that the infective agent belongs in the group of filterable viruses, though further work will be necessary to learn more of its exact nature. These observations present additional evidence that the presence of cells with nuclear inclusions in any lesion indicates that the injury is probably due to an infective agent belonging in the group of filterable viruses.

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EXPLANATION OF PLATE 33.

FIG. 1. Swollen epithelial cells containing nuclear acidophilic inclusions within a duct of the submaxillary gland of a full grown guinea pig. Magnification $\times 1700$.

FIG. 2. Duct cells of smaller size containing acidophilic nuclear inclusions. This illustration was made from a section of the submaxillary gland of a young pig, exact age not known. Duct cells of this size containing inclusions have been found only rarely. Possibly these cells are in process of transformation. Magnification $\times 1700$.

FIG. 3. Low power drawing from a section made from the submaxillary gland of a full grown guinea pig. Shows a duct swollen with epithelial cells containing nuclear inclusion bodies. There is a moderate degree of cellular reaction in the vicinity of the infected duct.

FIG. 4. Low power drawing of a section of the brain of a young guinea pig inoculated with an emulsion of the submaxillary gland of a full grown guinea pig. A well marked meningeal exudate is shown.

FIG. 5. High power ($\times 1700$) drawing of the cellular meningeal exudate seen in low magnification in Fig. 4.



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